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Joana Vanessa Cordeiro Melro Mourão

CONTRIBUTION OF BIOCIDES TO THE SELECTION OF *SALMONELLA* NON-TYPHOID RESISTANT TO ANTIBIOTICS: A MULTILAYERED APPROACH

Tese do 3.º Ciclo de Estudos Conducente ao Grau de Doutoramento em Ciências Farmacêuticas na Especialidade de Microbiologia

Trabalho realizado sob a orientação da Professora Doutora Patrícia Antunes (Faculdade de Ciências da Nutrição e Alimentação, Universidade do Porto, Portugal) e coorientação da Professora Doutora Luísa Peixe e Professora Doutora Carla Novais (Faculdade de Farmácia, Universidade do Porto, Portugal).

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É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE;

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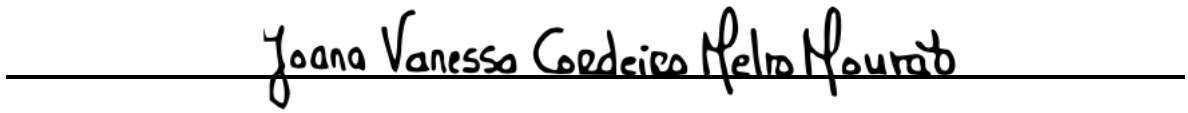

To my parents for teaching me to never give up

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ABSTRACT

Non-typhoidal *Salmonella* is a worldwide major foodborne zoonotic pathogen with a main reservoir in the intestinal tract of food-producing animals, from which it is readily transmitted to farm surrounding environments and the food chain. Expansion of successful multidrug-resistant (MDR) *Salmonella* serotypes and clones have been globally reported, but the factors imperative for their success, besides antibiotic resistance-ABR, remains scarcely explored. Tolerance to diverse antimicrobial non-antibiotic compounds (biocides) widely used in food-producing animals might constitute an adaptive feature to diverse environments and hosts. Among them are metals, often used in biocidal products (e.g. copper; silver-disinfectants, antiseptics/medicines or preservatives), in animal feed additives (e.g. copper, zinc) or in soil fertilisers/pesticides (e.g. arsenic, tellurium). Consequently, they can remain and accumulate in the animal-production setting and other environments, representing a long-term selective pressure potentially driving co-selection of metal-tolerant and/or ABR bacteria. In this context, for the elaboration of this thesis it was **hypothesized** that tolerance to metals/biocides contributes for the survival and emergence/persistence of particular MDR *Salmonella* serotypes and clones in diverse environments and hosts. The **main goal** was to assess the occurrence of acquired metal tolerance-Me^{T} genes as well as to characterize their associated genetic platforms and tolerance phenotype in different *Salmonella* serotypes and emergent clones. This assessment included 406 *Salmonella* isolates (2000-2014), belonging to 50 serotypes and diverse clones with different rates of frequency and emergence in the last 15 years. They were selected by source, antibiotic susceptibility phenotypes/genotypes, PFGE profiles and/or Sequence Types (ST) from a collection of ~2500 Portuguese isolates, and they were recovered in several Portuguese regions and different sources (humans, food products, food-animal production and the environment).

The search of acquired Me^{T} genes (PCR/sequencing) showed that those coding for copper (*pcoA-pcoD±silA-silE*), silver (*silA-silE*) and mercury (*merA*) tolerance were more frequent than those coding for arsenic (*arsB*) and tellurite (*terF*) tolerance. The occurrence of these diverse genes in isolates from variable sources, clones and serotypes suggests a genetic flow in different ecological communities and hosts. Of note are *Salmonella* belonging to recently emerging pig-associated serotypes/clones (MDR *S.* 4,[5],12:i:- and *S.* Typhimurium European clones/ST34 and *S.* Rissen/ST469) often isolated in the food chain and implicated in human infections in Europe/Portugal, which were more enriched in *sil±pco* genes than those associated with other food-animal settings (e.g. *S.* Enteritidis-poultry/eggs). The use of high copper concentrations allowed in feed for pigs comparing with other animals might have contributed for the selection and persistence of such emerging *sil±pco Salmonella*. Also, these isolates were more resistant to antibiotics than others without *sil±pco,* highlighting the importance of carrying diverse adaptive features to survival in environments contaminated with variable stressors as metals or antibiotics.

The characterisation of the genetic platforms carrying Me^{T} genes [genomic location (I-*Ceu*I/S1-PFGE/hybridization), plasmid analysis (plasmid-based replicon typing/sequencing) and conjugation/transformation assays] identified variable elements contributing for the co-dispersion

of Me^T and ABR genes in diverse *Salmonella* serotypes. Large plasmids of different major families (e.g. Inc A/C, R, FIIA, HI1, HI2, N, P, I1), some transferable and described to be widespread in *Enterobacteriaceae*, were found to carry biocide (*oqxAB*-quaternary ammonium compounds), Me^T (*sil±pco*, *merA*, *arsB*, *terF*) and ABR genes [e.g. *tet*(A), *sul1* and/or *sul3*-class 1 integrons] including clinically relevant ones ($bla_{CTX\text{-}M\text{-}9}$ and *aac(6')-Ib-cr/oqxAB*). Although some of these plasmids have already been described as carrying Me^T genes, we unveiled the relevance of IncFIIA and IncN types in the dispersion of such genes. The co-location of both Me^T and ABR in the same plasmids also alert for the importance of single horizontal transfer events for their co-dispersion among bacteria sharing the same ecosystems and subjected to diverse selective pressures. Variable Me^T genes (*sil±pco*, *merA, terF*) were also found in non-transferable chromosomal regions with other ABR genes [e.g. bla_{TEM}, *tet(B)*, *sul2*] among several *Salmonella* serotypes, but mostly in the recently emergent MDR *S.* 4,[5],12:i:- and *S.* Typhimurium European clones and *S.* Rissen. These data highlight the importance of clones environmental adaptation not only for their spread but also for maintenance of Me^T genes. Concerning *sil±pco* genetic environments, our results and an *in silico* genomic analysis showed that those clusters are often adjacent and mostly inserted in a Tn*7*-like element (Tn*6230*) with different levels of recombination potentially implicated in its genome fixation, particularly in recently emergent *Salmonella* serotypes/clones.

Phenotypic susceptibility assays to copper and silver (MIC_{CuSO4} and MIC_{AgNO3} in aerobic and anaerobic atmospheres by standard agar dilution method) showed that *Salmonella* isolates (different serotypes/clones; wild type and transconjugants) carrying the *sil* efflux system (chromosomal or plasmids) were associated with higher tolerance to $CuSO_4$ in anaerobiosis and to $AgNO_3$ after prior exposure to silver in aerobiosis. These data demonstrate the role of *sil* gene cluster for *Salmonella* adaptation to different metal stressors present in food-animal settings, particularly to overcome the more toxic Cu^+ occurring in reducing environments (e.g. animal gut, manure). Moreover, those modified methodological approaches in Cu/Ag tolerance assays were critical to identify isolates carrying *sil±pco* gene clusters and for the proposal of tolerance cut-offs to $CuSO_4/AgNO_3$, that can be used in further studies and allow comparison of data.

The **general conclusion** of this thesis is that the acquisition of metal tolerance genes, particularly to copper and silver, confers an advantage to clinically relevant multidrug-resistant *Salmonella* serotypes/clones by facilitating their better survival and persistence in environments contaminated with those metals across the food chain. The new information provided by this study might help to support the implementation of more efficient intervening measures associated with the use and/or accumulation of metals in diverse environments to prevent a wider expansion of foodborne zoonotic pathogens such as MDR *Salmonella* serotypes/clones or the emergence of new ones.

Keywords: *Salmonella*, Clones, Multidrug-resistance, Metals, Biocides

RESUMO

Salmonella não tifóide é um patogénico zoonótico de origem alimentar relevante a nível mundial. O seu principal reservatório é o trato intestinal dos animais, a partir do qual é facilmente transmitido para o ambiente de produção animal e cadeia alimentar. A expansão de serótipos e clones de *Salmonella* bem-sucedidos e que apresentam resistência a múltiplos antibióticos-MDR tem sido globalmente reportada. Contudo, outros fatores além da resistência a antibióticos-ABR que podem contribuir para esse sucesso permanecem pouco explorados. A tolerância a diversos compostos antimicrobianos não incluídos no grupo dos antibióticos (ex. biocidas), amplamente utilizados em produção animal, poderá constituir um fator adaptativo de *Salmonella* a diversos ambientes e hospedeiros. Entre estes compostos incluem-se os metais frequentemente utilizados em produtos biocidas (ex. cobre, prata-desinfetantes, antissépticos/medicamentos ou conservantes), aditivos na alimentação animal (ex. cobre, zinco) ou fertilizantes/pesticidas (ex. arsénio, telúrio). Na sequência do seu uso, os metais podem permanecer e acumular-se em diferentes ambientes, nomeadamente nos de produção animal, representando uma pressão seletiva a longo prazo que poderá levar à co-seleção de bactérias tolerantes a metais e/ou resistentes a antibióticos. Neste contexto, a **hipótese** desta tese foi de que a tolerância a metais/biocidas contribui para a sobrevivência e emergência/persistência de determinados serótipos e clones de *Salmonella* MDR em diversos ambientes e hospedeiros. O **principal objetivo** consistiu em avaliar a ocorrência de genes adquiridos de tolerância a metais-Me^{T}, caracterizar as plataformas genéticas em que se inserem e o fenótipo de tolerância em diferentes serótipos de *Salmonella* e clones emergentes. Foram incluídos 406 isolados de *Salmonella* (2000-2014) de 50 serótipos e diversos clones com taxas de frequência e de emergência variáveis nos últimos 15 anos. Estes isolados foram selecionados de uma coleção de ~2500 *Salmonella* de diversas regiões de Portugal com base na origem (humanos, alimentos, animais de produção e ambiente), fenótipos/genótipos de suscetibilidade aos antibióticos, perfis de PFGE e *Sequence Types* (ST).

A pesquisa de genes adquiridos de Me^{T} (PCR/sequenciação) mostrou que os que codificam para tolerância ao cobre (*pcoA-pcoD±silA-silE*), prata (*silA-silE*) e mercúrio (*merA*) foram mais frequentes do que os que codificam para tolerância ao arsénio (*arsB*) ou telúrio (*terF*). A ocorrência destes genes em isolados de diversas origens, clones e serótipos sugere um fluxo genético entre diferentes nichos ecológicos e hospedeiros. De destacar os serótipos/clones de *Salmonella* recentemente emergentes e associados a suínos (Clones Europeus/ST34 de *S.* 4,[5],12:i:- e *S.* Typhimurium, *S.* Rissen/ST469, todos MDR), frequentemente isolados na cadeia alimentar e implicados em infeções humanas na Europa/Portugal, mais enriquecidos em genes *sil±pco* do que os associados a outros animais de produção (ex. *S.* Enteritidis-aves/ovos). As elevadas concentrações de cobre permitidas nas rações de suínos comparativamente às de outros animais podem ter contribuído para a seleção e persistência destas *Salmonella* emergentes com *sil±pco*. Estes isolados também apresentaram maior resistência a antibióticos, destacando-se a importância da presença de vários fatores adaptativos para a sua sobrevivência em ambientes contaminados com diversos stresses como metais ou antibióticos.

A caracterização das plataformas genéticas portadoras de genes de Me^T [localização genómica (I-*Ceu*I/S1-PFGE/hibridação), análise de plasmídeos (PL) (PCR-*based replicon typing*/sequenciação) e ensaios de conjugação/transformação] identificou vários elementos associados à co-dispersão de genes Me^T e ABR em diversos serótipos de *Salmonella*. Detetaram-se plasmídeos de elevado tamanho e de diferentes famílias (ex. Inc A/C, R, FIIA, HI1, HI2, N, P, I1), alguns transferíveis e considerados amplamente distribuídos em *Enterobacteriaceae,* como sendo portadores de genes de tolerância a biocidas (ex. *oqxAB*-compostos de amónio quaternário), Me^T (ex. *sil±pco*, *merA*, *arsB*, *terF*) e ABR [ex. *tet(*A), integrões de classe 1-*sul1*/*sul3*], incluindo clinicamente relevantes (ex. $bla_{CTX\text{-}M\text{-}9}$, *aac(6')-Ib-cr*/*oqxAB*). Embora alguns desses plasmídeos tenham sido já descritos como portadores de genes Me^T, neste trabalho evidenciou-se a relevância dos IncFIIA e IncN na dispersão destes genes. A co-localização de genes Me^T e ABR nos mesmos plasmídeos reforça também a importância de eventos únicos de transferência horizontal para a sua co-dispersão entre bactérias que partilham os mesmos ecossistemas e sujeitas a diversas pressões seletivas. Vários genes Me^T (*sil±pco*, *merA, terF*) e ABR [ex. bla_{TEM}, *tet(B)*, *sul2*] foram também detetados em regiões cromossómicas não transferíveis em vários serótipos de *Salmonella*, mas principalmente nos clones Europeus de *S.* 4,[5],12:i:- e *S.* Typhimurium e *S.* Rissen, todos MDR e de emergência recente. Estes dados salientam a importância da adaptação dos clones a determinadas condições ambientais não só para a sua melhor propagação, mas também para a manutenção de genes Me^T. Os dados deste estudo juntamente com uma análise genómica *in silico* mostraram que os *clusters pco+sil* estão frequentemente adjacentes e inseridos em elementos Tn*7-like* (Tn*6230*), com diferentes níveis de recombinação possivelmente implicados na sua fixação genómica, particularmente em serótipos/clones de *Salmonella* de emergência recente.

Os ensaios de suscetibilidade ao cobre e à prata (CMI_{CuSO4} e CMI_{AgNO3} em aerobiose e anaerobiose pelo método standard de diluição em agar) mostraram que os isolados de *Salmonella* (diferentes serótipos/clones; selvagens e transconjugantes) portadores do sistema de efluxo *sil* (cromossoma ou plasmídeos) foram associados a uma maior tolerância a $CuSO_4$ em anaerobiose e a $AgNO_3$ após exposição prévia à prata em aerobiose. Estes dados contribuem para reforçar o papel do *cluster sil* na adaptação de *Salmonella* a diferentes metais presentes no ambiente de produção animal, particularmente para superar a forma mais tóxica Cu^+ presente em ambientes reduzidos (ex. intestino animal, estrume). A modificação metodológica destes ensaios de tolerância ao Cu/Ag foi crítica para identificar isolados com *sil±pco* e para propor valores *cut-off* de tolerância, permitindo a comparação criteriosa de dados quando utilizados em estudos futuros.

Os resultados obtidos permitem inferir como **conclusão geral** que a aquisição de genes Me^T, particularmente os que codificam para tolerância ao Cu/Ag, conferem uma vantagem a determinados serotipos/clones clinicamente relevantes de *Salmonella*, facilitando a sua melhor sobrevivência e persistência em ambientes contaminados com esses metais ao longo da cadeia alimentar. Este conhecimento poderá suportar a implementação de medidas de intervenção mais eficientes relativas à utilização e/ou acumulação de metais em diversos ambientes, prevenindo assim a expansão e/ou emergência de patogénicos zoonóticos como serótipos/clones de *Salmonella* MDR.

Palavras chave: *Salmonella*, Clones, Resistência a múltiplos antibióticos, Metais, Biocidas

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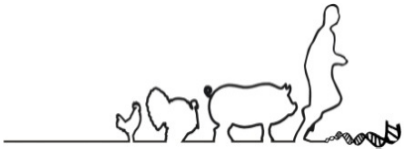
	Description
ABR	Antibiotic resistance
Ag	Silver chemical symbol
As	Arsenic chemical symbol
CDC	Centers for Disease Control and Prevention
CLSI	Clinical and Laboratory Standard Institute
Cu	Copper chemical symbol
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside triphosphates
eBG	eBurstGroups
ECDC	European Center for Disease Prevention and Control
ECOFFs	Epidemiological Cut-Off Values
EDTA	Ethylenediaminetetraacetic acid
EEA	European Economic Area
EFSA	European Food Safety Authority
EMA	European Medicines Agency
ESBL	Extended-Spectrum β-Lactamases
ESC	Extended-Spectrum Cephalosporins
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GI	Genomic Island
Hg	Mercury chemical symbol
HGT	Horizontal Gene Transfer
ICE	Integrative Conjugative Element
IR	Inverted Repeat
IS	Insertion Sequence
Kb	Kilobase
LGT	Lateral Gene Transfer
MCO	Multicopper oxidase
MDR	Multidrug Resistance
MH	Mueller-Hinton

MIC	Minimum Inhibitory Concentration
MGE	Mobile Genetic Element
MLST	Multilocus Sequence Typing
mM	Milimolar
NCBI	National Center for Biotechnology Information
PAI	Pathogenicity Island
PBPs	Penicillin Binding Proteins
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
PMQR	Plasmid-Mediated Quinolone Resistance
QACs	Quaternary Ammonium Compounds
QRDR	Quinolone-Resistance Determining Region
RFLP	Restriction Fragment Length Polymorphism
Rep	Plasmid-specific replication initiator protein
RNA	Ribonucleic Acid
RND	Resistance-nodulation-cell division
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
ST	Sequence Type
Te	Tellurium chemical symbol
USA	United States of America
WHO	World Health Organization



Chapter 1

Introduction

"It's not that I'm smart, it's just I stay with problems longer."

Albert Einstein

1.1. The foodborne zoonotic *Salmonella*

1.1.1. Clinical relevance, transmission dynamics and epidemiology

Salmonella enterica (henceforward *S. enterica*) infections are a worldwide major public health concern, being the foodborne illness salmonellosis caused by non-typhoidal *Salmonella* (serotypes other than *S.* Typhi and *S.* Paratyphi) (See box 1 for details). Currently, there are over 2500 identified *Salmonella* serotypes, although *S. enterica* subspecie I *enterica* includes all of the major serotypes that are pathogenic to humans (See box 1 for details) (1, 2). Salmonellosis is typically characterized by a self-limiting gastroenteritis syndrome (manifested as diarrhoea as main symptom, although fever, vomiting and abdominal pain or cramps can occur), with an incubation period between 4-72h, being death rare (<1%) (3-5). In healthy humans, the infectious dose is generally 10^6 to 10^8 bacterial cells, but lower number can cause disease in predisposing people, including infants and the elderly (4). Although uncommon, life-threatening invasive infections with bacteraemia (5-10% of infected persons) and/or other extra-intestinal infections may occur, affecting especially the risk groups (infants, young children, older people and the immunocompromised patients) (3-5). In severe cases, effective antimicrobial agents are essential, being the emergence of *Salmonella* resistant to critical antibiotics of concern (3, 4).

In industrialized countries, the main reservoir of non-typhoidal *Salmonella* is the gastrointestinal tract of warm-blooded animals, including food-producing animals from which it is readily transmitted to farm surrounding environments and the food chain (5-7). Therefore, although other sources (e.g. contact with animals/reptiles, environment or person-to-person) are recognized, the most relevant transmission to humans occurs mainly through the ingestion of contaminated food (e.g. eggs, poultry, pork, beef, dairy products and fresh produce), leading to a high global impact in human health (7). It was estimated that non-typhoidal *Salmonella* causes around 93.8 million of illnesses and 155.000 deaths each year worldwide (8). In the USA, the CDC estimated 1 million annual cases of foodborne salmonellosis, being associated with the largest number of hospitalizations (35%) and deaths (28%) compared with other foodborne microorganisms (9). In Europe, in the last year's salmonellosis has been the second most common zoonosis (82.694 confirmed cases and 20.4 cases per 100,000 populations in 2013) and the most frequent cause of foodborne outbreaks (22.5% of all cases), in spite of the reported decreasing trend scenario resulting from *Salmonella* control programmes (10).

Although different serotypes have been associated with salmonellosis, a limited number is responsible for most human infections, being *S. enterica* serotype Enteritidis (henceforward *S.* Enteritidis) the most frequent one in European Union (EU) (39.5% in 2013), USA (14.5% in 2012) and Canada (44.2% in 2013) followed by *S. enterica* serotype Typhimurium (henceforward *S.* Typhimurium) (20.2% in EU, 2013; 11.6% in USA, 2012; 8.5% in Canada, 2013) (10-12). *S.* Enteritidis is commonly associated with poultry and products thereof, while *S.* Typhimurium has a wider species range, including pigs and cattle as well as poultry (13). Therefore, foods of animal origin, including contaminated poultry products (eggs, egg products, poultry and turkey meat), but also pork have been considered the main vehicles of *Salmonella* infection (10, 14, 15).

In recent years, with the implementation of *Salmonella* control programmes mainly along poultry/egg production (e.g. in the EU and USA), changing trends in foodborne salmonellosis and associated serotypes were observed, with the expansion of previously less common serotypes (e.g. increase in reported cases of *S. enterica* Typhimurium monophasic variant – henceforward *S.* 4,[5],12:i:-) causing human infections through food chain and with origin in different food-animal species. Importantly, the increasing occurrence of these serotypes has been associated with certain subtypes, designated clonal lineages or clones (characterized by typing methods of reference as Pulsed-Field Gel Electrophoresis or Multilocus Sequence typing) (See box 2 for details) (16), which in turn exhibit resistance to multiple antimicrobial agents (2, 3, 17, 18). With the increasing globalization of foodstuffs like poultry and pig meat, which are two of the most consumed and increasing globally traded meat products (19, 20), and changes in food-animal production and in consumer behaviours (e.g. increased consumption of fresh produce) new problems might arise regarding salmonellosis control. An overview of the role of food-animals, mainly poultry/eggs and pig (the most common sources of human infection) on salmonellosis at a global scale and in the persistence/emergence of specific *Salmonella* serotypes/clones is provided on the next topic.

BOX 1 - Serotyping and Phage Typing

Salmonella spp. is classified in ≈2500 serotypes based on the classical Kauffmann–White-Le Minor scheme, with the majority of them (≈1500) belonging to *S. enterica* subsp. I *enterica*, a group of greatest clinical relevance due to its common association with humans and warm-blooded animals. In this technique, specific agglutination reactions are observed between adsorbed antisera that contain specific antibodies ("factors"), and the corresponding antigens when present within either surface lipopolysaccharides (LPS, O-antigens) or two alternatively expressed phases of the flagellar antigens (proteins, H-antigens) (Figure 1) (1, 21). Based on the type of antigens detected each serotype is represented by an antigenic formulae written as O:H1:H2 (e.g. antigenic formulae 1,4,[5],12:i:1,2 defines *S.* Typhimurium) (1). In addition to classical/conventional serotyping, others methods such as PCR or genoserotyping (a microarray assay) can also be used for the identification of the most common reported *Salmonella* serotypes through detection of the genes coding surface O and H antigens (16). Phage typing is carried out by infecting a specific *Salmonella* serotype (the most common Typhimurium and Enteritidis), with a number of specific bacteriophages, leading to a typing scheme based on the susceptibility to lysis (22).

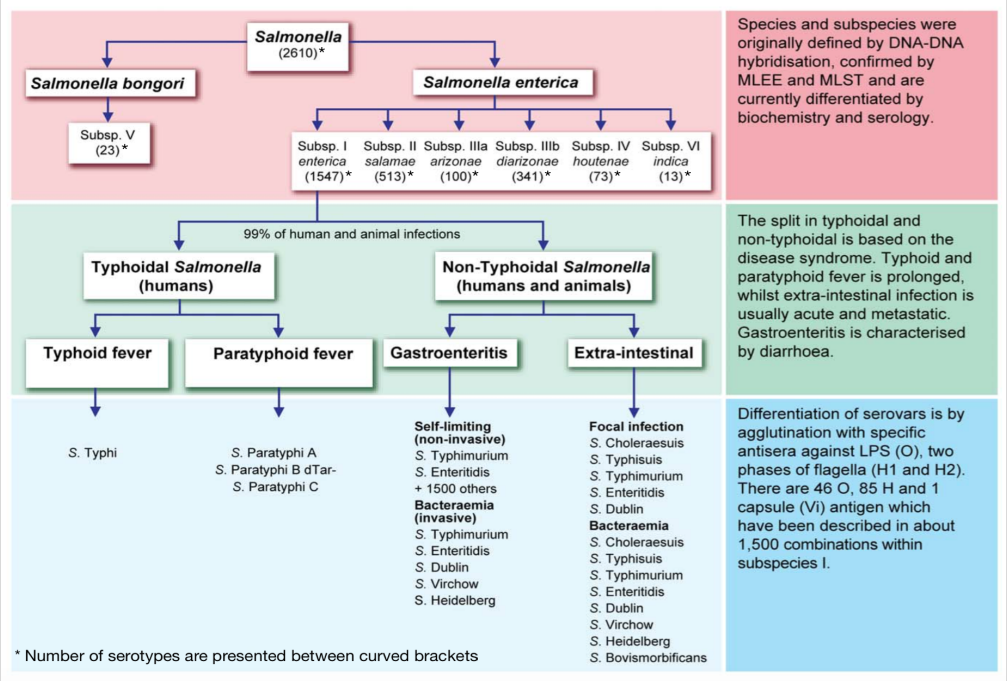


FIGURE 1 – Classification of *Salmonella* by classical/conventional typing methods and disease syndrome [adapted from reference (21)].

BOX 2 - Molecular typing methods – PFGE, MLST and others

Pulsed-Field Gel Electrophoresis (PFGE) has a higher discriminatory power comparing for example with serotyping and phagetyping and is widely used for characterizing *Salmonella*, particularly in outbreak investigations. This method uses restriction enzymes (e.g. *Xba*I as primary and *BIn*I or *Spe*I as additional enzymes are currently used) to cut bacterial DNA in specific restriction sites, being these fragments separated through a gel matrix in a size-dependent manner by carefully orientated electrical pulses (16). The analysis of *Salmonella* DNA fingerprint on the gel is then used to discriminate PFGE-profiles and clones.

Multilocus Sequence Typing (MLST) is based on sequencing of internal fragments of seven housekeeping genes, usually distributed around the chromosome. For each of the seven genes, different sequences (alleles) occur. To obtain an MLST type, the seven gene sequences are deposited and forwarded to the online database (http://mlst.warwick.ac.uk/mlst/) and an allelic profile or Sequence Type (ST) is returned (Figure 2). Additionally, two or more STs that share six of the seven alleles that defined the ST were further grouped in genetically closely related clusters, named eBurstGroups (eBGs) (21).

BOX 2 - Molecular typing methods – PFGE, MLST and others (cont.)

Other molecular typing methods are also recently used for the characterization of *Salmonella* outbreaks, such as Multiple-Locus Variable number tandem repeat Analysis (MLVA) or Whole Genome Sequencing (WGS) (16). The MLVA might have an improved discriminatory power compared with PFGE, thus can be used as a complement of this technique (16). It is based on measurement of length of variable number of tandem repeat (VNTR) loci by PCR amplification and electrophoresis, using this information to create a genotype to distinguish between isolates of the same serotype (16). WGS is based on the full sequencing of a bacterial genome, thus achieving the highest discriminatory power between the several methods. Nevertheless, some challenges have been reported for WGS such as the storage, interpretation, annotation and harmonization of the huge amount of information (16).

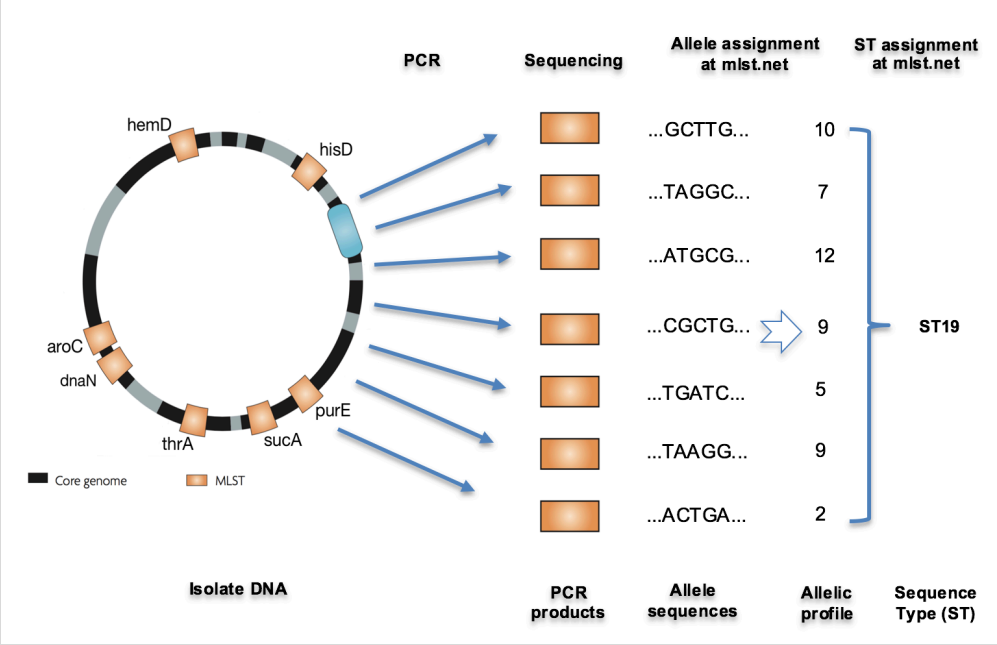


FIGURE 2 – MLST sequential scheme for obtaining *Salmonella* ST (http://mlst.warwick.ac.uk/mlst/dbs/Senterica). An example is given for ST19 achievement, which is characteristic of *S.* Typhimurium and *S.* 4,[5],12:i:-.

1.1.2. Non-typhoidal *Salmonella* serotypes in poultry/eggs and pig products

Animal populations, in particular chicken, but also turkey and pigs, are frequently colonized with *Salmonella* without manifestation of detectable symptoms (sub-clinical infections/healthy carriers). They are colonized by horizontal (e.g. contaminated feed and water) and vertical transmission at primary production level (6, 7, 23). Additionally, in early stages of the production line (e.g. slaughter process of the animals, egg collecting or milking) cross-contamination can also occur (24, 25). The presence of *Salmonella* in asymptomatic healthy animals, that may excrete *Salmonella* continuously or intermittently, is suggested as the main risk factor for allowing bacteria to be easily

transmitted vertically in table eggs (through infected chickens), and in animal meat to humans. Animal faeces may also contaminate farmland, surface water flow and vegetables if they are fertilized with untreated animal manure (Figure 3) (13, 23, 26).

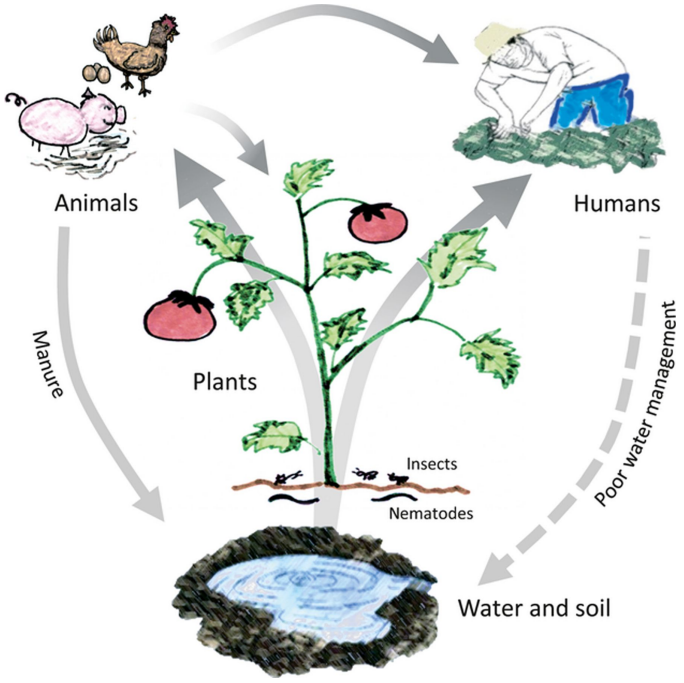


FIGURE 3 – Infection routes and dissemination of *Salmonella* within the ecosystem [reprinted with permission from reference (26)].

In Europe, it is assumed that the observed reduction in salmonellosis cases (32% between 2008 and 2012) is mainly due to implemented successful *Salmonella* control measures (e.g. surveillance, biosecurity, sanitation/disinfection, feed and water additives, vaccination) (25, 27, 28), targeting poultry (including breeding flocks of *Gallus gallus*, broilers and breeding and fattening flocks of turkeys) and eggs production (including laying hens and eggs) (10, 29-32). Those measures are focused in particular serotypes, such as *S.* Enteritidis, *S.* Typhimurium (including the monophasic variant *S.* 4,[5],12:i:-), *S.* Infantis, *S.* Virchow and *S.* Hadar, considered of public health significance (6, 10, 13, 27, 32). This scenario led to the achievement of reduction of *Salmonella* rates for poultry populations in most EU countries and lower non-compliance regarding *Salmonella* in poultry/eggs products (6, 10). Moreover, these decreasing contamination rates in European raw poultry products are in agreement with those recently observed in industrialized countries from other geographical regions applying pathogen reduction programmes, such as the USA (27, 33, 34). It is worth to mention, that by 2000´s high incidence of *Salmonella* in poultry products was reported in the EU, with rates higher than 50% for several countries (35). In the 2013 zoonosis EFSA/ECDC report involving

data from European countries, as in previous years, *Salmonella* was most frequently reported, although at low levels, in fresh turkey (5.4%) and fresh broiler meat (3.5%), in comparison with eggs (0.1%) or fresh pig meat (0.7%) (10). Despite the highest incidence being detected in poultry meat, consumption of contaminated eggs and pig meat still remain the most important sources of food-borne *Salmonella* outbreaks (10). In fact, using quantitative source attribution models the higher number of human salmonellosis cases in Europe was attributable to eggs (65% in 2011 and 17% in 2012) and pigs (28% in 2011 and 56.8% in 2012) comparing with broilers (2.4% in 2011 and 10.6% in 2012) and turkeys (2.6% in 2011 and 4.5% in 2012) (14, 15). However, diverse surveys targeted to detect *Salmonella* in poultry and pig products in developing countries, some with expansion of the food-animal industry, still detected high percentages of positive samples, ranging from ~13-39%-poultry/~39%-pig in South America (36-38), ~35%-poultry/~14-40%-pigs in Africa (39-42) and ~35-50%-poultry/~30-40%-pig in Asia (43-47). Those differences possibly reflect diverse poultry production husbandry practices and absence of control measures along the food chain, highlighting the importance of the extensive international trade of animals and products thereof for *Salmonella* spread (6).

Worldwide data about *Salmonella* serotypes prevalence in humans and in the diverse range of foodstuffs have contributed to establish an epidemiological link between salmonellosis and poultry or pig products, with diverse serotypes overlapping between humans and poultry (chicken and turkey) or pig meat (Figure 4). In EU, recent changes in the frequency of *Salmonella* serotypes causing human infections were reported, which in some cases were in line with those occurring in poultry and pig meat, reflecting the importance of food chain transmission by these type of products to humans (Figure 4). Nevertheless, interpretation of these data should be cautious, owing to limitations in the number of human and food isolates serotyped each year. Of particular relevance is the decrease in *S.* Enteritidis human cases (19% reduction between 2011 and 2013 in EU, following the observed reduction in human salmonellosis), a serotype typically associated with poultry meat and eggs consumption, in line with a decreasing trend in poultry and poultry products pointed by different studies (11, 27, 48-50). Increasing occurrences of other serotypes implicated in human infections (e.g. *S.* Infantis, *S.* Stanley, *S.* Kentucky) related to poultry meat (chicken and turkey) have been reported in the EU (Figure 4). However, one major difference in the serotype pattern between humans and poultry is related to *S.* Typhimurium and its monophasic variant, both frequently associated with human salmonellosis cases, but less common in poultry meat, being pigs and pig meat the main source (10). In USA and Canada, other serotypes like *S.* Heidelberg and *S.* Kentucky have emerged as predominant serotypes in poultry and have also been implicated in human salmonellosis, beyond *S.* Enteritidis (11, 12, 27, 33, 34, 51-53).

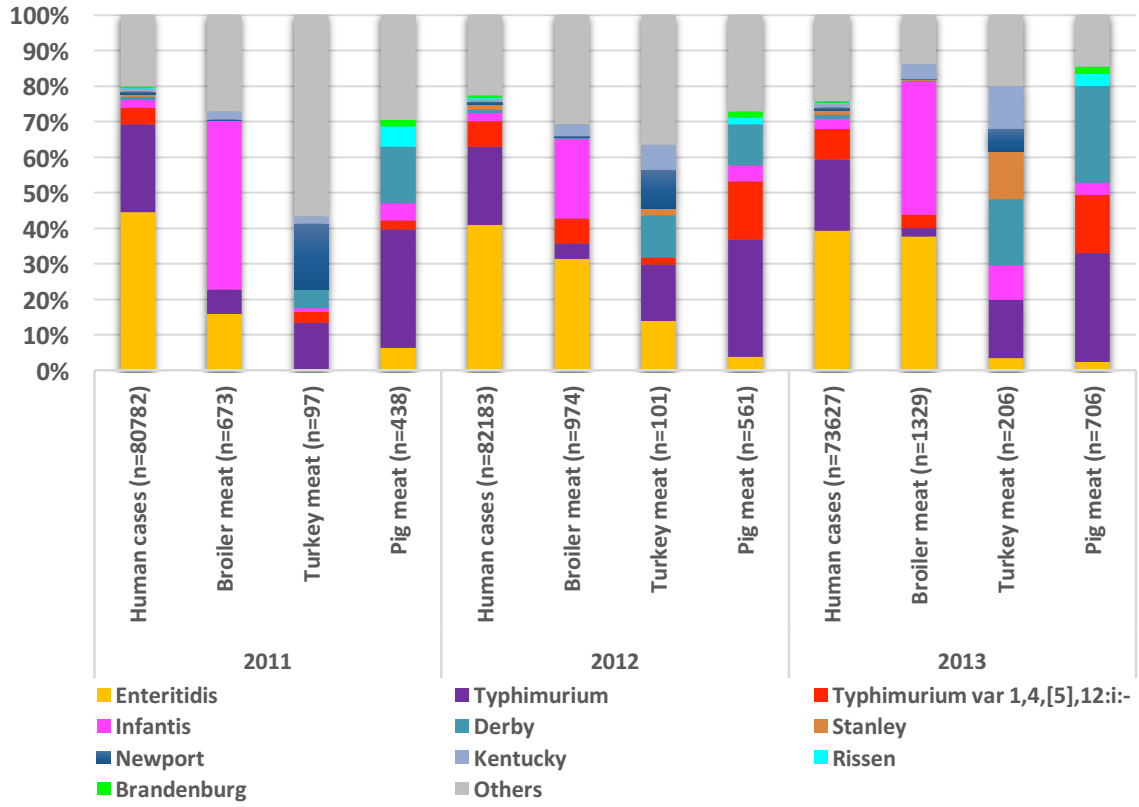


FIGURE 4 – Distribution of the major serotypes of non-typhoidal *Salmonella* associated with human cases (salmonellosis), poultry and pig meat in EU, 2011 to 2013. The data were obtained from EFSA reports: humans (2011 to 2013) and turkey/broiler/pig meat (2013) (10); turkey/broiler/pig meat (2011 to 2012) (54, 55) (Last accessed 1 November 2015). The percentages were calculated based on the total number of serotyped isolates per type of meat or human salmonellosis cases.

The shift in *Salmonella* serotypes related to poultry and poultry production has been associated with the spread of certain clones, with most of them being responsible for large outbreaks (Table 1). For instance, *S.* Infantis increase (26.5% in human cases between 2011 and 2013 accounting for the fourth most common serotype and the most reported in broilers and broiler meat) (10), has been associated with the spread of several clones of broiler origin in diverse European countries, including the dominant Hungarian clone (56-58). Also, *S.* Stanley showed an increase since 2011 with a peak in 2012, being the sixth most common human serotype and one of the three most common in turkey meat, together with *S.* Derby and *S.* Kentucky in 2013 (10). A large *S.* Stanley outbreak caused by a new clone (novel PFGE-type), was linked with the consumption of turkey meat, and is still circulating in the European food market (at least since 2011), with a considerable risk of becoming endemic in the poultry production chain in Europe (59-61). In the USA, *S.* Heidelberg in particular has been identified as one of the top human and

poultry serotypes, with several clones implicated in diverse large multistate outbreaks resulting from the consumption of contaminated chicken or turkey products (27, 53). The spread and the global persistence of serotype *S*. Kentucky reflect other particular situation related to the increased globalization of travel and food/animal trade in different geographical regions. This serotype has been associated with a worldwide (Europe, Africa, and Asia) spread of a particular epidemic clone (*S*. Kentucky ST198-X1), recovered from several livestock reservoirs, particularly poultry farms, with chicken and turkey implicated as the potential major human infection vehicles (18, 62-65).

TABLE 1 – *Salmonella* outbreaks and emerging clones linked to poultry (including eggs), pig and products thereof.

Serotype (clone designation when applied)	Source	Year(s)[a]	Country	No. of[b]:		Reference(s)
				Cases	Deaths	
4,[5],12:i:- (European clone)	Pork products	2006	Luxembourg	133	1	(66)
4,[5],12:i:-	Banquet pot pies (chicken or turkey)	2007	USA	272	0	CDC, 2015[c]
	Dried pork sausage	2010	France	69	0	(67)
	Dried pork sausage	2011	France	337	0	(68)
	Pork	2015	USA	188	0	CDC, 2015[c]
Derby	Raw fermented pork	2013-2014	Germany	145	NS	(69)
Enteritidis	Eggs, scrambled	2007	USA	81	0	CDC, 2015[c]
	Eggnog	2008	USA	18	0	CDC, 2015[c]
	Eggs, scrambled	2009	USA	59	0	CDC, 2015[c]
	Eggs and poultry	2009	United Kingdom	152	2	(70)
	Shell eggs	2010	USA	1939	NS	CDC, 2015[c]
	Raw eggs	2014	Europe (multi-country)	287	1	(71)
	Chicken	2015-	USA	5	0	CDC, 2015[c]
	Chicken	2015-	USA	15	0	CDC, 2015[c]
Hadar	Turkey burgers	2010-2011	USA	12	0	CDC, 2015[c]
	Live poultry	2012	USA	46	0	CDC, 2015[c]
Heidelberg	Broiled chicken livers	2011	USA	190	0	CDC, 2015[c]
	Ground turkey	2011	USA	136	1	CDC, 2015[c]
	Chicken	2012-2013	USA	134	0	CDC, 2015[c]
	Chicken	2013-2014	USA, Puerto Rico	634	0	CDC, 2015[c]
	Chicken	2014	USA	9	0	CDC, 2015[c]
Infantis (Hungarian clone)	Broilers and chicken	2004-2009	Europe	NA	NS	(56-58)
Infantis (Israel clone)	Broiler chickens	2007-2009	Israel	NA	NS	(50)
Infantis	Raw pork products	2013	Germany	267	1	(72)
	Raw poultry products	2015-	Canada	98	0	PHAC, 2015[f]
Kentucky (Sequence Type 198-X1 clone)	Chicken and turkey	2002-2013	Europe, Africa, Asia	NA	NS	(62-64)
Montevideo	Live poultry	2012	USA	93	1	CDC, 2015[c]
Stanley	Turkey	2011-2013	Europe	710	NS	(59, 61)
Typhimurium	Raw egg mayonnaise	2009	Australia	71	0	AGDH, 2015[d]
	Salami	2010	Denmark	20	0	(73)
	Duck eggs	2010	Ireland	24	0	FSAI, 2015[e]
	Live poultry	2013	USA	356	0	CDC, 2015[c]

[a], "year-" include ongoing reported outbreaks.
[b], Estimated number of cases only when outbreaks were reported. NA, not applicable in emerging clones. NS, not specified.
[c], CDC, 2015. *Salmonella*. Reports of selected *Salmonella* Outbreak Investigations. Available at: http://www.cdc.gov/salmonella/outbreaks.html [accessed December 2015].
[d], AGDH, 2015. Annual report of the OzFoodNet network, 2009. Available at: http://www.health.gov.au/internet/main/publishing.nsf/Content/cda-cdi3404b.htm [accessed December 2015].
[e], FSAI, 2015. Large outbreak in recent years linked to *Salmonella* in duck eggs. Available at: https://www.fsai.ie/14092010.html [accessed December 2015].
[f], PHAC, 2015. Outbreak of *Salmonella* infections under investigation. Available at: http://www.phac-aspc.gc.ca/phn-asp/2015/salmonella-infantis-eng.php [accessed December 2015].

With the implementation of control programs in poultry/eggs production and with the subsequent *Salmonella* prevalence reduction, the role of pork products has been enhanced, being nowadays one of the most important sources of human salmonellosis at global or the EU level (10, 74). In fact, using quantitative source attribution models, EFSA estimated that around 28% (14) to 56.8% (15) of the human salmonellosis cases are attributable to pigs. Concerning pig production, control programs are not compulsory at the moment by the Regulation (EC) No. 2160/2003 of the European Parliament and Council, which are primarily focused on *Salmonella* control at poultry production (29). Most of national monitoring programs for *Salmonella* on pig meat and products thereof are based on sampling at the slaughterhouse (e.g. animal faeces, lymph nodes, organ or tissue samples, carcase or nasal swabs) and/or at the processing or cutting plants (10). In fact, EFSA reported a significant percentage of *Salmonella* in pig samples (8.1%) but also at the herd (14.9%) and slaughter (30.0%) batch levels (10). With a similar scenario, the United Kingdom in a baseline survey also reported a high prevalence of *Salmonella* in faecal samples (30.5%), rectal swabs (24.0%) and carcase swabs (9.6%) of slaughter pigs (75). The presence of *Salmonella* in all steps of pig production chain, which may contribute to meat contamination, alert to the need for implementing proper and effective measures to detect and control *Salmonella* serotypes with public health significance, in the pig setting and particularly at the primary farm production level (25, 76).

In Europe, *S.* Typhimurium has been the most frequently reported serotype in pigs (47.8%) and pig meat (30.7%) in line with its frequent association with human salmonellosis (20.2% in human cases in 2013 accounting for the second most common serotype) (Figure 4). On the other hand, the second most common serotype in pig meat, *S.* Derby (14.8% in pigs and 27.1% in pig meat) showed also an increase in human salmonellosis cases (0.9% in 2012 to 1.1% in 2013), although mainly due to an outbreak associated with raw fermented pork (Table 1; Figure 4) (10). Similarly, in a European baseline study reporting a high prevalence (31.8%) of *Salmonella*-positive holdings with breeding pigs, the two most common serotypes in breeding and production holding were *S.* Derby (29.6% and 28.5%) and *S.* Typhimurium (25.4% and 20.1%) (77, 78). These two serotypes were also the most frequently isolated in clinical and non-clinical cases in pigs in USA (27, 79).

Noteworthy, over the last years in several European countries, including Portugal, it has been reported an increase in the prevalence (68.8% between 2011 and 2013) of *S.* 4,[5],12:i:-, a pig-related serotype, ranking nowadays third in frequency (Figure 4) (10, 80, 81). In the USA and Canada, the emergence and spread of this serotype associated with human infections, has also been observed, being the fifth most common in both regions (11, 79, 82, 83). *S.* 4,[5],12:i:- isolates have been responsible for several recent worldwide

large outbreaks (Table 1) (66-68, 84) and food animals, particularly pigs, were pointed out as its reservoir and their derived products as the main infection vehicles (77, 78, 80, 85). This serotype is, since 2011, targeted by EU actions to detect and control *Salmonella* serotypes of concern (32), requiring further surveillance and mitigation strategies, including its epidemiological success characterization. Two major *S.* 4,[5],12:i:- clones were recognised and were previously designated as "European" and "Spanish". The "European" clone has been circulating in diverse geographical regions of Europe and has been frequently associated with DT120 and DT193 phage types (85-88), whereas the "Spanish" clone, spread in the Iberian Peninsula since 2002, was mostly associated with DT104/U302 phage types (89, 90). As a result of the worldwide increase of *S.* 4,[5],12:i:- clones causing human infections (86) and its increasing expansion in pig populations (85), the monophasic variant is currently considered an emerging epidemic serotype (80, 86).

Other pig-related serotype is *S.* Rissen, recently reported as having an increase in their frequency globally (10). It is now in the TOP20 of the most reported serotypes associated with human salmonellosis in Europe but, in Portugal, this serotype ranked fourth in frequency between 2002 and 2013 (10, 81). *S.* Rissen is usually associated with a predominant PFGE-type clone, resistant to multiple antibiotics and frequently associated with *sul1*-typical or *sul3*-atypical class 1 integrons (24, 91-94). This serotype has been reported in samples from European pigs, pork and surrounding environments. For example, *S.* Rissen was detected in lairage, pig carcasses and intestines samples (17.4%) from Belgium (95), in pig liver and colon content (4.6%) from Italy (92), in pigs and farm environments (7.5% to 15.38%) from Northern Ireland (96) or in pig carcass (10.1%) from The Netherlands (94). Although, in the Iberian Peninsula an increasing frequency (~ >20%) of this serotype have been reported (48, 91, 93, 97-99). In fact, in a European baseline survey study, a high incidence of *S.* Rissen in breeding and production pig holdings has been found in Portugal (40% and 22.4%, respectively) and Spain (25% and 29.7%, respectively), being the 1st or 2nd most frequently reported (77, 78). Other non-European regions, such as USA (100-102) and Thailand (101, 103-106) have also been found this serotype in pig abattoirs and retail meat.

These and other examples of multi-country/multistate outbreaks or clonal expansion of *Salmonella* infections linked to poultry and pig meat (Table 1) serves as a reminder of the importance of acting upon any *Salmonella* contamination in the food chain and monitoring to detect the emergence of any serotype or new clone. This scenario also alerts for the need of inclusion of those serotypes or particular clones, considered of public health significance, in control and surveillance programmes (13, 27, 60).

1.1.3. *Salmonella* adaptive features to food-animal setting

Salmonella serotypes and clones associated with human infections and with an enhanced ability to colonize several food animals, able to persist across the food chain (e.g. primary production on-farm, slaughter operations, equipment's and meat handlers, retail meat) with efficient transmission and rapid spread, are of public health relevance (17, 18, 56, 107). Although the exact mechanisms of their persistence and spread in poultry and pig production are still largely unknown, recent studies focusing on emergent poultry and pig-associated *Salmonella* serotypes/clones unveiled specific features (e.g. enhanced colonization/virulence, metal tolerance), other than antibiotic resistance, that could provide a significant advantage both in the environment and in the host (poultry/pig/human) (27, 108, 109).

Recent studies found the presence of diverse virulence genes in *S.* Typhimurium (108, 110) and in specific clones of its monophasic variant *S.* 4,[5],12:i:- strains circulating in Europe (80, 85, 90, 111) associated with a better adaptation to the food-animal host (110). These genes encoded for proteins related to improved intestinal colonization (e.g. *clpB* - chaperone), adhesion (e.g. *csgA*, *fimA*, *stbD* - fimbrial operons) or infection (e.g. *sopA*, *avrA*, *sseI* - effector genes that function to alter host cell physiology and promote bacterial survival in host tissues; *marT* – transcriptional regulator that activates the expression of an extracellular matrix adhesin, the MisL; and *fliB* - flagellar operon) (110). Also, a recent study showed that *S.* Typhimurium DT193 and *S.* 4,[5],12:i:-, two serotypes with increased adaptation to faecal pH and organic acid supplementation, were associated with a long-term survival in porcine faeces compared to others such as *S.* Derby and *S.* Bredeney (112). In fact, the persistence of these two pig-associated *Salmonella* serotypes may be important for a better transmission by faecal-oral routes in pig herds (112).

In poultry associated serotypes other examples could be cited. In Israel, a comparison between the pre-emergent and the emergent *S.* Infantis clones showed in the latter the presence of a megaplasmid (hybrid plasmid pESI belonging to IncI1 and IncP-1α families of ~280Kb) associated with an increased tolerance to stress factors (e.g. mercury and oxidative stress) and virulence/pathogenicity (e.g. enhanced biofilm formation, adhesion and invasion into avian and mammalian host cells) (113). Also, a genomic study of several predominant *Salmonella* serotypes from Canadian broiler chickens showed the presence of multiple features related to pathogenicity (e.g. genes encoding adhesins, flagellar proteins, iron acquisition systems, type III secretion system-T3SS) and stress tolerance (e.g. metal and antiseptic tolerance genes; better acid-stress response) (51). *S.* Heidelberg, including ground turkey outbreak isolates, carried phages and plasmids with diverse virulence factors (e.g. P2-like phage-*sopE1* gene, IncX-T4SS), which could play a

role in their virulence (a serotype highly associated with invasive infections), colonization and persistence (a poultry-associated serotype) (27, 53). In *S.* Kentucky, the acquisition of an *E. coli* ColV virulence plasmid (IncFIB/FIIA families) with genes encoding particular virulence features (e.g. afimbrial adhesins and colicins), was also associated with enhanced colonization of the chicken cecum and persistence in the avian extraintestinal environment, particularly in a dominant avian clonal type (27, 114).

These features can play a role in the successful spread of emergent and virulent serotypes/clones that could contribute in short time to replacing other *Salmonella*. Moreover, those emergent *Salmonella* serotypes are usually enriched with antimicrobial (antibiotics, metals or biocides) resistance determinants conferring multidrug-resistance/tolerance (27, 51, 53, 112, 113), which is currently one of the major public health concerns (discussed in the next topics).

1.2. Antibiotic-resistant non-typhoidal *Salmonella* infections and the food-animal link

1.2.1. Use of antibiotics and the growing burden of antibiotic resistance

Several national and global entities, such as World Health Organization (WHO), have identified antibiotic resistance as one of the major threats to human health, also linked to food safety via food ingestion, direct contact with animals or environmental spread (115, 116). This grim scenario results from the extraordinary adaptation of bacteria to selective pressures caused by the intensive use of antibiotics, mainly in non-human applications. In Europe, antibiotics are mainly used in livestock production for animal health in veterinary medicine as, since 2006, they were banned as growth promoters. In other regions, the real contribution of the use of antibiotics for animal health *versus* the use as growth promoters continues largely unknown (Table 2) (18, 117-119).

TABLE 2 – Antimicrobials approved for use in food-producing animals [adapted with permission from reference (120)].

Antimicrobial families/classes	Antimicrobial	USA			Europe		Portugal[c]
		Used in C, P, S[b]	Available for growth promotion	Human use	Used in C, P, S[b]	Human use	Used in C, P, S[b]
Penicillins	Amoxicillin	C, P, S		X	C, S	X	C, P, S
	Ampicillin	C, P, S		X	C, S	X	C, P, S
	Cloxacillin	C		X	NL	X	C
	Penicillin (Procaine)	C, S	X	X	C, S	X	C, S
1st generation cephalosporins[a]	Cephalexin	C, P, S		X	C, P, S	X	C
	Cefalonium	C, P			C, P		C
	Cephapirin	C			C		C
	Cefazolin	C, P		X	C, P	X	C
	Cefacetrile	C			C		NA
2nd generation cephalosporins[a]	Cefuroxime	C		X	C	X	NA
3rd generation cephalosporins[a]	Cefoperazone	C, P		X	C, P	X	C
	Ceftiofur	C, P, S			C, S		C, S
	Ceftriaxone	C, P, S		X	C, P, S	X	NA
4th generation cephalosporins[a]	Cefquinome	C, P, S			C, S		C, S
Quinolones	Danofloxicin	C			C		C
	Enrofloxacin	C, S			C, P, S		C, P, S
Aminoglycosides	Apramycin	S	X		NL		C, P, S
	Gentamicin	C, P, S		X	P, S	X	C, P, S
	Neomycin	C, P, S	X	X	C, P, S	X	C, P, S
Tetracyclines	Chlortetracycline	C, P, S	X		C, P, S		C, P, S
	Oxytetracycline	C, P, S	X	X	C, P, S	X	C, P, S
	Tetracycline	C, P, S		X	C, P	X	C, P
Macrolides	Oleandomycin	C			P, S	X	NA
	Tilmicosin	P, S	X		NL		C, P, S
	Tylosin	C, P, S	X		C, S		C, P, S
	Erythromycin	C, P, S		X	C, P, S	X	C, S
Polypeptides	Bacitracin	C, P, S	X	X	Withdrawn	X	Withdrawn
Flavofosfolipid	Bambermycin	C, P, S	X		Withdrawn		Withdrawn
	Flavomycin	P	X		Withdrawn		Withdrawn
Quinoxaline	Carbadox	P, S	X		Withdrawn		Withdrawn

TABLE 2 - *Continued*

Antimicrobial families/classes	Antimicrobial	USA			Europe		Portugal[c]
		Used in C, P, S[b]	Available for growth promotion	Human use	Used in C, P, S[b]	Human use	Used in C, P, S[b]
Polypeptides	Colistin/Polymyxin B	C, P	X	X	C, P, S	X	C, P, S
Phenicol	Florfenicol	C, S			C, S		C, S
Lincosamines	Lincomycin	C, P, S	X	X	C, P, S		C, P, S
	Pirlimycin	C			NL		C
Novobiocin	Novobiocin	C, P		X	C, P	X	NA
Aminocyclitol	Spectinomycin	C, P, S		X	NL	X	C, P, S
Diterpene	Tiamulin	S	X		P, S		P, S
Triamilide	Tulathromycin	C, S			NL		C, S
Streptogrammin	Virginiamycin	P, S	X		Withdrawn		Withdrawn
Sulfonamides	Sulfadimethoxine	C, P, S	X		P		C, P, S
	Sulfamethazine	C, P, S	X		S		C, P

[a], Although extensively implemented in the past, FDA and EU have now controlled the off-label and unapproved use of cephalosporins (especially, extended-spectrum cephalosporins) in poultry, cattle and pigs (120).
[b], C, cattle; P, poultry; S, swine; NA, not available; NL, not listed in the references used by Seiffert et al (120).
[c], All the approved antimicrobials are considered veterinary medicinal products subject to medical prescription (121).

In fact, in 2012, the quantities of active substances of antimicrobials sold in 26 EU/European Economic Area (EEA) countries for animals (7982 tonnes or average 144.0 mg/kg), specially in chickens and pigs, were higher than that applied in humans (3400 tonnes or average 116.4 mg/kg) (122). Similarly, in the USA about 13.569,037 kilograms of antimicrobial active ingredient were sold in 2011 for food-animals comparing with the 3.289,176 kilograms reported for human medicine (123, 124). However, the greatest uncertainty about current antibiotic use in livestock is in the low- and middle-income regions/countries, such as Africa or the Asiatic continent, where a rise in antibiotic consumption is estimated (125, 126). A recent global study indicated that some of those countries (e.g. China, Brazil, Mexico and India) were the largest consumers of antibiotics for livestock, and after the application of statistical models a projection estimates that this will rise by two-thirds until 2030 (62.300 tonnes in 2010 to 105.600 tonnes in 2030), mainly due to an increase in livestock product consumption and the shift from extensive (i.e. small-scale) to large-scale intensive farming systems (i.e. industrial-scale) (125, 126).

The most frequently used antibiotic classes in food-producing animals were tetracycline's, sulphonamides, and penicillin's and, although 3rd-, 4th-generation cephalosporins and fluoroquinolones consumption was much lower for animals than for humans a small increase has been observed over the last years (18, 120, 122, 124, 127-129). Several classes of antimicrobials may not be fully metabolised by the human and animal bodies, and a large amount may be excreted in faeces or urine and enter wastewater treatment facilities (118). The slow rate of degradation of some antibiotics and the use of treated and untreated sewage as fertiliser can also lead to the accumulation of antimicrobials in diverse environments/hosts, including in food-animals and consequently in the food-chain production (Figure 5) (118). These subinhibitory concentrations may provide a constant selection pressure for maintenance of particular pathogenic bacteria, such as non-typhoidal *Salmonella* serotypes or clones carrying antibiotic resistance genes and other adaptive features associated with survival or improved host and environmental colonization (18, 118).

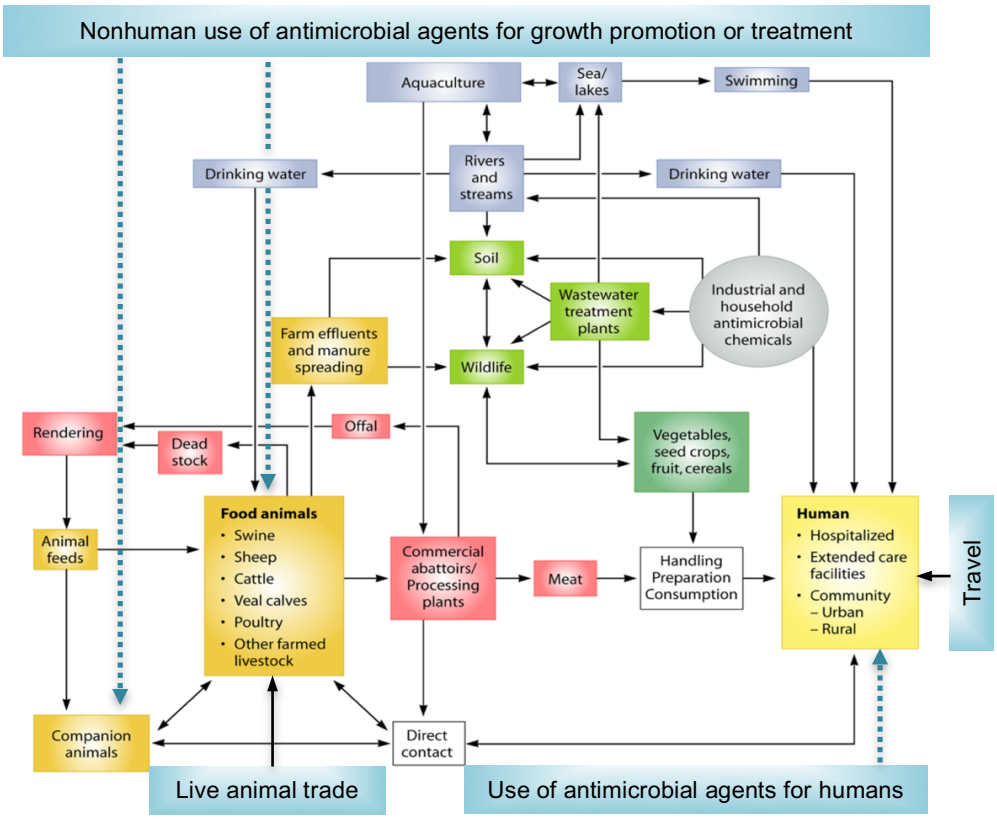


FIGURE 5 – Potential routes for dissemination of antimicrobial-resistant bacteria and resistance genes. Blue dotted arrows represent the selective pressures introduced by the use of antimicrobial agents [adapted from references (118, 130)].

Currently, the emergence and spread of *Salmonella* isolates presenting antimicrobial resistance (R) (see box 3 for details) or multidrug resistance[1] (MDR) profiles, especially to clinically relevant antibiotics for treating invasive infections, are of concern (3, 17). In fact, the raise of antimicrobial resistance represents a huge economic burden both within the EU and the USA (e.g. $365.000.000 in medical costs per year) (79, 131, 132). Since resistance to older antibiotics (e.g. ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole) has been increasing for many years, recommended treatment options for invasive infections included fluoroquinolones (ciprofloxacin) and extended-spectrum cephalosporins (3, 4). However, resistance to those "critically important antibiotics for human health" is emerging, leading to increase severity, morbidity and mortality of the disease and the need for the use of last-line antimicrobials (e.g. carbapenems) in therapy (18, 133). The contribution of food-animals for the global burden of antibiotic-resistant *Salmonella* serotypes/clones, particularly to β-lactams and quinolones are given in the next topics.

[1] Isolates resistant to at least one antibiotic are considered resistant (R) and to three or more antibiotics from different families are considered multidrug-resistant (MDR) (132).

BOX 3 - Antimicrobial resistance: concepts

Susceptibility of a microorganism to a particular antibiotic can be quantified by phenotypic methods such as the determination of the minimum inhibitory concentration (MIC), which is the lowest concentration of an antimicrobial agent that completely inhibits the growth of bacterial cells (117, 134, 135). The use of clinical breakpoints (based on clinical outcome data, MICs distributions, accepted dosing, pharmacokinetic/pharmacodynamic data) are extremely useful for clinicians to access the likelihood of therapeutic failure/success in human patients (135). Based on these clinical breakpoints, the isolate is defined as 1) resistant, 2) intermediate or 3) susceptible, accordingly to annual published guidelines and recommendations such as Clinical & Laboratory Standards Institute (CLSI - http://clsi.org/) or European Committee on Antimicrobial Susceptibility Testing (EUCAST - http://mic.eucast.org/Eucast2/). Contrarily the use of epidemiological cut-off values (ECOFFs) can be applied to distinguish between organisms without and with phenotypically expressed resistance mechanisms for a species and a drug in a defined test system. In this case microorganism can be categorized in wild type (WT-absence of acquired and mutational resistance mechanisms to the drug in question) and Non-Wild Type (NWT-presence of an acquired or mutational resistance mechanism to the drug in question). ECOFFs are defined as the MIC values that separate the upper limit of the wild type population from those isolates that have a phenotypically expressed resistance (Figure 6) (134, 135).

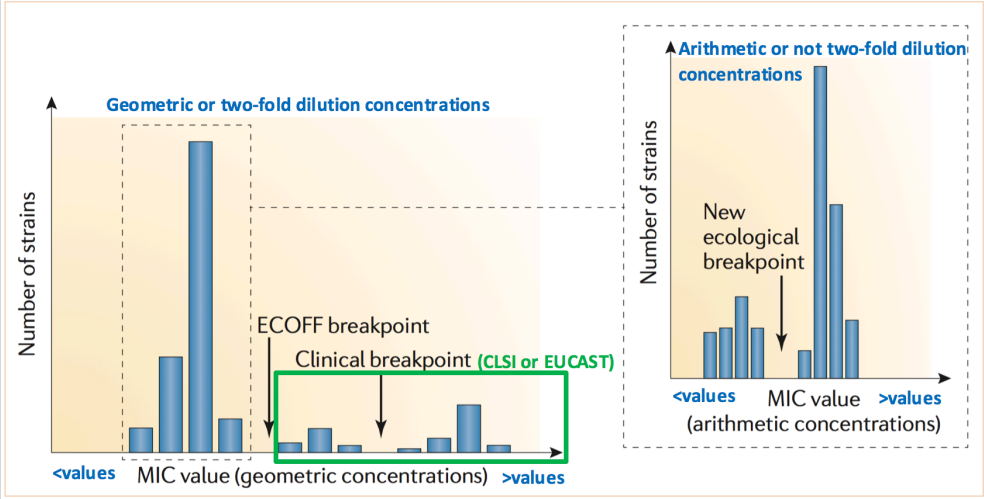


FIGURE 6 – Clinical and epidemiological breakpoints of susceptibility to antibiotics [adapted with permission from reference (134)].

1.2.2. The contribution of food-animals for the global burden of antibiotic-resistant *Salmonella*

For several decades, the contribution of food-animals as a reservoir of antimicrobial resistance with impacts in human health has been controversial, but accumulating evidences linking particularly the poultry and pig production with human disease have been reported, namely involving non-typhoidal *Salmonella* (136-138). The first evidence is the close association between the use of antimicrobial agents and the occurrence of resistance. In fact, as previously mentioned, regular use of antibiotics (mainly for animal health and in some countries as growth promoters), a practical associated with modern intensive food-animal production, has been considered the main

driver for the development of antibiotic resistance in zoonotic bacteria, such as *Salmonella* (120, 136, 138). For example, licensing of the fluoroquinolone enrofloxacin for animal use, especially in poultry, in the 1990s led to an increase of *S.* Typhimurium DT104 with decreased susceptibility to ciprofloxacin in animal, food and humans (7). On the other hand, an experimental study has shown that administration of tetracycline to pigs colonized with tetracycline-resistant *S.* Typhimurium DT104 was associated with elevated shedding of resistant *Salmonella* compared with untreated pigs (139). Some findings have also suggested a link between resistance to nitrofurans in human *Salmonella* and the food chain, suggesting that its illegal use in poultry industry might have contributed to the selection and persistence of *S.* Enteritidis in poultry, and consequently to human salmonellosis in Portugal (140). More recently, a voluntary withdrawal of ceftiofur by the poultry producers in Canada was correlated with a decrease in the occurrence of ceftiofur resistant *S.* Heidelberg (one of the most common serotypes associated with salmonellosis in this country) from both human infections and retail poultry, with an increase of the resistance levels after reintroduction of use (52).

Further evidence for the impact of food-animal production on human health problems associated with antimicrobial resistance in *Salmonella* is the correlation between different reservoirs (e.g. humans and poultry or pig) obtained from systematic surveillance data. In the last EU report, *Salmonella* resistant or MDR to commonly used antimicrobials (e.g. ampicillin-36.1%, sulphonamides-35.7% and tetracycline-34.5%) was frequently detected in humans (R=50%; MDR=31.8%) and animals, especially poultry [broilers (MDR=56%) and turkeys (MDR=73%), with 5-98% resistance to ampicillin, 5-85% to sulphonamides and 4-85% to tetracycline] and pigs (MDR=37.9%, with 72-91% resistance to ampicillin, sulphonamides and tetracycline), but also in derived meat products (18, 125). Also, in the USA, high levels of resistance and MDR in animal's/retail meat, mainly chicken (R=58.2%; MDR=9.1%/R=59.6%; MDR=26%), turkey (R=64.3%; MDR=28.6%/R=77.4%; MDR=39.6%) and pigs (R=25.6-33.5%; MDR=9-17.7%/R=45.8%; MDR=33.3%), and moderate levels in humans (R=19.2%; MDR=9.8%) were found in the integrated NARMS report (141).

Those data about MDR are extremely worrying due to the possible role of diverse antibiotics in the co-selection of *Salmonella* strains resistant to clinically relevant antibiotics, such as fluoroquinolones and extended-spectrum cephalosporins. In EU, relatively low levels of *Salmonella* non-susceptible ('clinically' resistant and 'intermediate' resistant categories combined) to ciprofloxacin (3.8%) and "microbiological" resistant (non-wild type by epidemiological cut-off values - ECOFFs) to cefotaxime (1.4%) were observed in humans (18). Moreover, the highest levels of "microbiological" resistance to these critically important antimicrobials were detected in broiler meat (68% ciprofloxacin

and 10.1% cefotaxime, with 0.3% of co-resistance to both antibiotics) and turkey meat (73.4% ciprofloxacin and 4.7% cefotaxime) and low levels in pig meat (3.9% ciprofloxacin and 0.9% cefotaxime), suggesting a greater contribution of the poultry production chain to the human burden (18). In fact, it was reported that the highest levels of resistance to ciprofloxacin were more common in *S.* Enteritidis, *S.* Infantis and *S.* Kentucky, three serotypes commonly associated with poultry meat (18). Also, in the USA, in spite of a decreasing trend since 2009, cephalosporin's-ceftriaxone resistance levels remain low in humans (2.5%) and in pig/pork (2.3-2.4%/0%), but still high in turkey/retail ground turkey meat (7.1%/9.4%) and chicken/retail chicken meat (7.3%/19.7%), especially in *S.* Heidelberg a poultry-associated serotype (15% in humans, 29% in retail ground turkey and 17.9% chicken) (141). In middle-income countries such as in the Asiatic continent, high levels of *Salmonella* non-susceptible to ciprofloxacin (15-48%) and cephalosporins (38% ceftriaxone) were observed in humans (142, 143). These data are in agreement with several studies that documented a high prevalence of resistance to fluoroquinolones (>22.5% ciprofloxacin) and cephalosporins (12.5->23.4% ceftriaxone and 26.6% ceftazidime) mainly in poultry meat from South Korea and China (44, 45). In these countries the high rates of antimicrobial resistance detected reflect the levels of antimicrobial consumption in livestock, including for growth promotion, an area that remains largely unregulated (45, 126, 143).

A third indication of the significant impact of antibiotic-resistant *Salmonella* (see box 4 for details) contaminating these foodstuffs on human health are the diverse reports demonstrating the same acquired antibiotic resistance mechanisms, particularly to the clinically important cephalosporins and fluoroquinolones, and/or the transmission of clinically relevant MDR *Salmonella* clones from food-animals to humans (17, 120, 137, 144).

BOX 4 - Antibiotic resistance mechanisms in *Salmonella*

Several antibiotic resistance mechanisms have been described, such as production of enzymes that inactivate antimicrobial agents through degradation or structural modification, reduction of bacterial cell permeability to antibiotics, activation of antimicrobial efflux pumps, and modification of cellular drug targets (145). Some of those mechanisms can be intrinsic (the innate ability of a bacterial species to resist/tolerate to the activity of a particular antimicrobial agent through its inherent structural or functional characteristics) to the bacteria or acquired via mutations in chromosomal genes and by horizontal gene transfer (Figure 7) (145, 146). As previously mentioned, *Salmonella* resistance to older antibiotics (e.g. chloramphenicol and trimethoprim-sulfamethoxazole) presented below, but even of greater concern to those clinically relevant (e.g. cephalosporins and fluoroquinolones) has become a public health issue.

- Phenicols modifying enzymes - Phenicols are a group of bacteriostatic compounds. Among them is chloramphenicol, a potent inhibitor of protein synthesis, able to prevent the formation of peptide bonds upon binding to the 50S ribosomal subunit (147). One of the resistance mechanisms is associated with drug target modification due to the production of acetyltransferases. An example may be that encoded by *cat* genes, which are able to acetylate both hydroxyl radicals of the chloramphenicol molecule, and according to its structure can be of type A or B (145). Phenicol resistance by efflux pumps results from the production of specific transport proteins such as those encoded by *floR* and *cmlA* genes, able to efflux florfenicol and chloramphenicol or only the second one, respectively (145).
- Resistance to trimethoprim and sulphonamides by modification of the cellular drug-binding target - These two agents inhibit the synthesis of nucleic acids by blocking the formation of purine and pyrimidine, interfering in consecutive stages of the folic acid biosynthetic pathway (147, 148). In *Salmonella* the genes responsible for sulphonamide resistance (*sul1*, *sul2* and *sul3*) encode altered dihydropteroate synthases enzymes that have a reduced affinity for the antibiotic. Likewise, trimethoprim resistance is possible due to production of alternative dihydrofolate reductases that have a reduced affinity for the antibiotic. To date, more than 30 genes identified in resistance to trimethoprim are divided according to their structure in *dfrA* (e.g. *dfrA12* and *dfrA17* frequently reported in *Salmonella*) and *dfrB* types (145, 148).

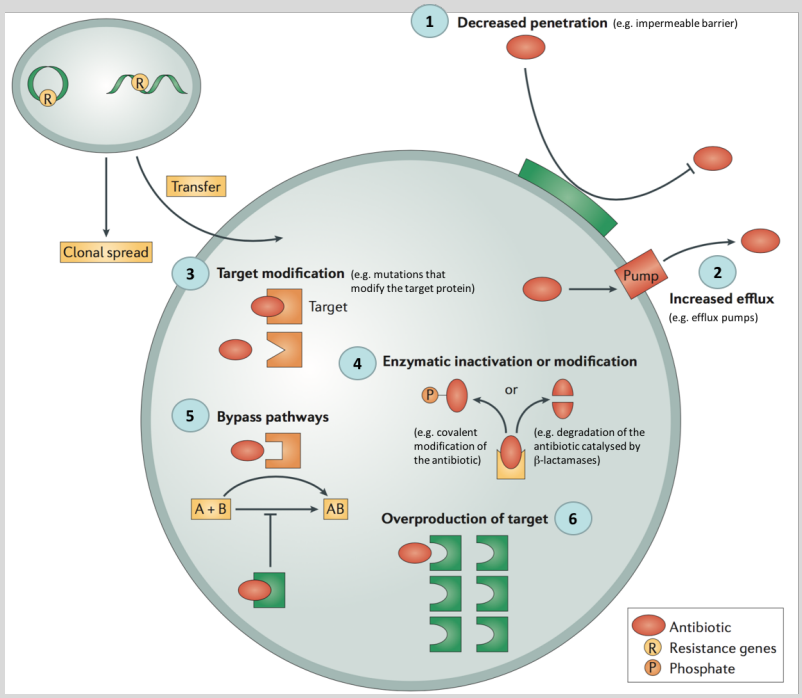


FIGURE 7 – Examples of antimicrobial resistance mechanisms in bacteria [adapted with permission from reference (149)].

1.2.2.1. Resistance to extended-spectrum β-lactams in *Salmonella*

β-lactams (see box 5 for details), particularly extended-spectrum cephalosporins (ESC), are the most widely used bactericidal antibiotics in clinical practice worldwide, including as a therapeutic option for human salmonellosis (18). However, over the last decade resistance to β-lactams has been extensively reported in *Enterobacteriaceae*, including in *Salmonella*, becoming a serious public health problem that requires full attention and appropriate management (18, 120). The most common mechanism of β-lactams resistance in *Salmonella* is through the production of enzymes, known as β-lactamases, capable of degrading the β-lactam ring, thereby inactivating their antimicrobial activity (145).

BOX 5 - Classification and mechanism of action of β-lactams

This class of antibiotics can be divided in four different groups: penicillin's, cephalosporins, carbapenems and monobactams. Some β-lactams have a narrow antimicrobial spectrum (penicillin's, first- and second-generation cephalosporins) being active mainly in Gram-positive bacteria, while others have a extended-spectrum (third-, fourth- and fifth-generation cephalosporins and carbapenems), acting on both Gram-negative and Gram-positive bacteria (150, 151). They are characterized by the presence of a β-lactam ring in their structure, acting by mimicking and thereby inactivating bacterial transpeptidases, such as penicillin binding proteins (PBPs), which are involved in the biosynthesis of peptidoglycan layer, necessary for cell wall formation (Figure 8) (147).

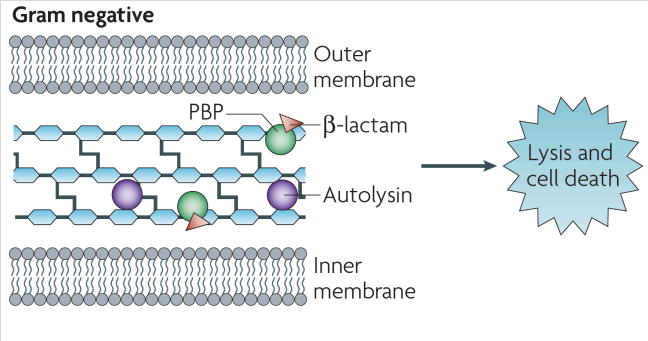


FIGURE 8 – β-lactams target interactions and associated cell death mechanisms [adapted with permission from reference (147)].

Due to their ability to hydrolyse critical important antibiotics in human and veterinary medicine, such as extended-spectrum β-lactams (especially third- and fourth-generation cephalosporins and carbapenems) three main important groups of β-lactamases are of special interest in *Salmonella*: extended-spectrum β-lactamases (ESBLs), AmpC β-lactamases and more recently carbapenemases (2, 120, 137, 152, 153).

The most frequent group, ESBLs[2], are generally acquired by horizontal gene transfer, arose by point mutations in the sequences of narrow-spectrum plasmid-mediated β-lactamase genes (e.g. *bla*$_{TEM-1}$ and *bla*$_{SHV-1}$), and include derivatives of the TEM (Greek patient "Temoneira") and SHV ("Sulfhydryl reagent variable") enzymes. The most prevalent group CTX-M (Cefotaximase Munich)-type enzymes have been associated with a mobilization process of chromosome-encoded *bla* genes from the environmental *Kluyvera* spp. (137, 150, 151, 154). A wide range of clinically important *Salmonella* serotypes (e.g. Enteritidis, Typhimurium, 4,[5],12:i:-, Heidelberg, Infantis, Kentucky, Virchow) mainly recovered from poultry and pigs (animal/food), have been associated with the worldwide dissemination of ESBLs (Table 3). By far, the most common genes found in poultry and/or poultry meat samples associated with resistance to extended-spectrum cephalosporins were those coding for CTX-M-1, CTX-M-2, CTX-M-9, CTX-M-15 and also TEM-52 enzymes (Table 3) (137). In pig and pork samples stands out the increasing report of CTX-M-15-producing *S.* Enteritidis and *S.* Virchow enzymes mainly in the Asiatic continent, although with a much lower frequency than that found in poultry (155). Also, the emergent *S.* 4,[5],12:i:- European clone has been recently identified as a producer of ESBLs such as CTX-M-1 enzymes mainly in UK and Germany, highlighting the role of this clonal lineage for the maintenance of this serotype in pigs (156, 157). Several studies have demonstrated the presence of some of those *Salmonella* clones carrying ESBLs in both human and food-animal isolates, suggesting an evidence of transmission along the food chain. For example, in several European countries, spread of *S.* Virchow producing CTX-M-2 or CTX-M-9 and *S.* Infantis producing TEM-52 have been found among poultry and humans (158-166).

The second most common group of β-lactamases in *Salmonella*, are the plasmid-encoded AmpC[3] cephalosporinases, being enzymes less well reported than ESBLs (137, 167). Within this group the most prevalent enzyme is CMY-2, having the broadest geographic spread and frequently found in diverse *Salmonella* serotypes mainly recovered from poultry and poultry meat, but also pig and pork products (Table 3). Of concern is *S.* Heidelberg, one of the top MDR serotypes in both poultry and human (frequently associated with invasive infections) in North America, presenting increasing resistance to cephalosporins associated with *bla*$_{CMY-2}$ (53, 168). Also MDR *S.* Typhimurium, the most

[2] The original definition of ESBLs is restricted to functional class 2be/molecular class A β-lactamases, being inhibited by clavulanic acid and possessing a hydrolytic activity against extended-spectrum cephalosporins (150). ESBLs can be classified into different families, according to their amino acid sequence (http://www.lahey.org/studies/).

[3] AmpC β-lactamases (Ambler class C) hydrolyses efficiently penicillin's and oxyimino-cephalosporins (including cephamycins) and are not inhibited by clavulanic acid (167). These genes encoded originally for chromosomally (cAmpC) inducible (e.g. present in *Citrobacter* spp., *Enterobacter* spp. and *Serratia* spp.) and non-inducible enzymes (e.g. *E. coli* and *Shigella* spp.). Later, they were mobilized to transmissible plasmids (pAmpC) and nowadays have been reported in other Gram-negative bacteria usually poorly expressing or lacking chromosomal *bla*$_{AmpC}$ genes, such as *Salmonella* (167).

prevalent pig-associated serotype and the second most common in human infections, has been increasingly associated with the AmpC *bla*$_{CMY-2}$ mainly in pig and pork from non-EU countries but also from Belgium (169-171).

In addition to the ESBL/AmpC-producing *Salmonella*, production of carbapenemases (e.g. metallo-β-lactamase such as VIM or NDM) present a major challenge for antimicrobial chemotherapy, since these bacteria are usually resistant to almost all β-lactams (including carbapenems) and most of them are not inhibited by classical β-lactamase inhibitors (152, 153). Recently, plasmid-mediated carbapenem-resistant bacteria from food-animals have been reported, alerting for a new public health problem with indefinable risks (120, 153). Although the prevalence of acquired carbapenemases is still low in *Salmonella* (152), some studies reported carbapenemases in strains recovered from humans (e.g. *bla*$_{KPC-2}$, *bla*$_{NDM-1}$, *bla*$_{OXA-48}$, *bla*$_{OXA-204}$, *bla*$_{VIM-1/-2}$), with most of them arising from endemic regions (e.g. Algeria, Egypt, India, Libya, Tunisia) (153, 172-179), wild animals (e.g. *bla*$_{NDM-1}$) (180) and food-animals (181). One of the first reports on livestock farms were on two fattening pig farms and one broiler farm in Germany, where a *bla*$_{VIM-1}$ gene (in an IncHI2 plasmid) was detected on *S.* Infantis strains from the environment and pig faeces (181, 182). In fact, this study demonstrates the ability of this clinically important serotype to be transmitted from farm environments to food-animals and potentially to humans via contaminated food (152, 153).

TABLE 3 - *Salmonella* serotypes/clones carrying extended-spectrum (ESBLs) and plasmid-encoded AmpC β-lactamases recovered from poultry, pigs and products thereof.

***Salmonella* serotype**	**ESBL or AmpC[a] enzyme (no. isolates)**	**Source**	**Concomitant presence in humans[b]**	**Country(ies)/year(s)**	**Non-β-lactam resistance phenotype[c]**	**Genetic element PL – Inc group**	**Reference (s)**
Agona	CTX-M-1 (n=1)	Poultry (caecum)		The Netherlands/2006	SUL-TET	PL – I1	(165, 166)
	CTX-M-2 (n=2)	Turkey carcass		Brazil/2008	CIP-NAL-STR-SXT-TET	PL – P	(183)
		Poultry		Brazil/2011	NR	PL – NR	(184)
	CTX-M-8 (n=3)	Poultry		Brazil/2008, 2010	NR	PL – NR	(184)
	TEM-52 (n=3)	Poultry		Belgium/2001, 2004	(-)	PL – I1	(162)
	CMY-2 (n=5)	Turkey		Germany/2005	(-) or NAL	PL – I1	(185)
Anatum	CMY-2 (n=4)	Pig		USA/2008-2011	TET	PL – I1-γ	(186)
Bareilly	ACC-1 (n=6)	Poultry	Yes	The Netherlands/2001-02	(-)	PL – NR	(187)
Blockley	TEM-52 (n=6)	Poultry, poultry meat	Yes	The Netherlands/2001-02	(-) or SPT-SUL-TMP	PL – NR	(187)
Bovismorbificans	CTX-M-1 (n=1)	Pig faeces		UK/2009	SUL	PL – I1-γ	(157)
Brackenridge	CTX-M-14 (n=1)	Poultry		Brazil/2011	NR	PL – NR	(184)
Braenderup	ACC-1 (n=2)	Broiler		The Netherlands/2001	(-)	PL – NR	(187)
		Broilers		The Netherlands/2006	(-)	PL – NT	(165)
	SHV-12 (n=1)	Poultry		Italy/2006	(-)	PL – NR	(188)
Bredeney	CTX-M-1 (n=1)	Turkey		USA/2010	NR	PL – N	(189)
	CMY-2 (n=1)	Turkey		Canada/1999	SUL-TET	PL – A/C	(190)
Derby	CTX-M-1 (n=2)	Healthy pigs		Belgium/2009	NR	NR	(170)
	TEM-52(n=1)	Poultry		Belgium/2001	(-)	PL – I1	(162)
Enteritidis	CTX-M-2 (n=1)	Poultry		Brazil/2004	NR	NR	(184)
	CTX-M-9 (n=1)	Broiler		Spain/2000-04	STR-SUL-SXT-NAL-TET	NR	(160)
	CTX-M-14 (n=5)	Imported chicken meat		China/2004	NAL	PL – NR	(191)
		Poultry		China/2012-13	CHL-CIP-ENR-FFC-NAL-OLA- SXT-(AMK-GEN-LVX-TET)	PL – HI2, F, N or B/O	(192)
	CTX-M-15 (n=36)	Chicken meat, faeces or disease	Yes	Korea/2009	STR-SUL-TET-(GEN-NEO)	PL – FII	(193)
		Chicken		South Korea/2009-10	NR or GEN-STR-SUL-TET	PL – FII	(194)
		Pigs		Korea/2012-13	GEN-NAL-NEO-STR-	HI2	(155)

TABLE 3 - *Continued*

***Salmonella* serotype**	**ESBL or AmpC[a] enzyme (no. isolates)**	**Source**	**Concomitant presence in humans[b]**	**Country(ies)/year(s)**	**Non-β-lactam resistance phenotype[c]**	**Genetic element PL – Inc group**	**Reference (s)**
					TET		
	SHV-12 (n=2)	Poultry		Italy/2006	TET	NR	(188)
		Broiler		Portugal/2012-13	CHL-CIP-SUL-NAL-TET	NR	(195)
Emek	CMY-2 (n=1)	Poultry		Brazil/2011	NR	NR	(184)
Essen	CTX-M-15 (n=1)	Diseased chicken		Korea/2009	GEN-STR-SUL-TET	PL – FII	(193)
Gaminara	CTX-M-14 (n=1)	Poultry		Brazil/2009	NR	NR	(184)
Give	CTX-M-2 (n=1)	Poultry		Brazil/2009	NR	NR	(184)
Hadar	CTX-M-14 (n=1)	Poultry		Brazil/2005	NR	NR	(184)
Havana	CTX-M-1 (n=1)	Broiler		Portugal/2012-13	SUL-TET	NR	(195)
	CMY-2 (n=2)	Broiler		Portugal/2012-13	(-)	NR	(195)
Heidelberg	CTX-M-1 (n=1)	Broiler carcass		Portugal/2012-13	SUL-TMP	NR	(195)
	CTX-M-2 (n=14)	Chicken		Venezuela/2005-07, 2008	CIP-GEN	PL – NR	(196)
		Poultry		Brazil/2009, 2011	NR	NR	(184)
	CTX-M-14 (n=1)	Poultry		Brazil/2011	NR	NR	(184)
	SHV-2 (n=1)	Retail chicken meat		Canada/2007	GEN-STR-SUL	PL – I1	(197)
	CMY-2 (n=47)	Chicken or chicken retail	Yes	Canada/2001-04	CHL-GEN-KAN-STR-SUL-SXT-TET	PL – A/C, I1	(168)
		Porcine		Canada/1999-2007	(CHL-SUL-TET-SXT)	PL – A/C, I1	(169)
		Poultry		Canada/1999-2007	(CHL-KAN-SUL-TET-SXT)	PL – A/C, I1	(169)
		Turkey, chicken		USA/2007	(-) or KAN-TET-(CHL-GEN-STR-SUL)	PL – I1, A/C, N	(198)
		Chicken food		USA/2002, 2004, 2010	(CHL-GEN-KAN-STR-SUL-TET)	PL – A/C, I1	(53)
		Poultry		Brazil/2011	NR	NR	(184)
Indiana	CTX-M-14 (n=22)	Poultry		China/2012-13	ENR-NAL-SXT-(AMK-CHL-CIP-FFC-GEN-LVX-OLA-TET)	PL – HI2, A/C, F, PL – N, P-1α, B/O	(192)
		Duck		China/2009-10	CIP-NAL	PL – N	(199)
	CTX-M-24 (n=29)	Chicken		China/2008-09	AMK-CHL-FFC-GEN-NAL-OLA-RIF-STR-SXT-(TET)	PL – HI2	(200)
	CTX-M-27 (n=3)	Duck		China/2009-10	CIP-NAL	PL – HI2, N	(199)
	CTX-M-65 (n=17)	Poultry		China/2012-13	CHL-NAL-OLA-SXT-(CIP-ENR-FFC-GEN-	PL – HI2, F, N	(192)

TABLE 3 - *Continued*

***Salmonella* serotype**	**ESBL or AmpC[a] enzyme (no. isolates)**	**Source**	**Concomitant presence in humans[b]**	**Country(ies)/year(s)**	**Non-β-lactam resistance phenotype[c]**	**Genetic element PL – Inc group**	**Reference (s)**
					LVX-TET)		
		Chicken		China/2012	GEN-AZI	PL – NR	(201)
		Chicken		China/2011	CHL-CIP-GEN-NAL-SXT-TET	PL – NR	(202)
		Pork		China/2012	GEN-AZI	PL – NR	(201)
		Pig		China/2011	CHL-CIP-GEN-NAL-SXT-TET	PL – NR	(202)
	TEM-52 (n=1)	Broilers		The Netherlands/2006	(-)	PL – NT	(165)
	DHA-1 (n=3)	Chicken faeces		South Korea/2006-07	SUL-TMP-(APM-NAL-NEO-STR-TET)	PL – NR	(203)
Infantis	CTX-M-1 (n=31)	Poultry (caecum or unknown)		The Netherlands/2006	SUL-TMP	PL – I1	(165, 166)
		Broiler chicken, broiler meat	Yes	Italy/2012-14	NAL-CIP-SUL-TET-TRI-(GEN-KAN-STR)	PL – P (pESI-like)	(204)
	CTX-M-2 (n=3)	Poultry		Brazil/2005	NR	NR	(184)
		Retail Chicken Products		Japan/2002-03	(-)	NR	(205)
	TEM-20 (n=2)	Retail Chicken Products		Japan/1997, 2003	(-)	NR	(205)
	TEM-52 (n=14)	Poultry	Yes	Belgium/2001-05	(-)	PL – I1	(162)
		Broiler		Belgium/2004	(-)	PL – I1	(163)
		Poultry (caecum or unknown)		The Netherlands/2006	(-)	PL – I1	(165, 166)
		Chicken		Japan/2004-06	(-)	PL – NR	(206)
		Retail Chicken Products		Japan/2000, 2003	(-)	NR	(205)
	CMY-2 (n=50)	Retail Chicken Products		Japan/1997, 1999-03	(-)	PL – NR	(205)
		Healthy pigs		Japan/2007-08	CHL-STR-SUL-TET	PL – NR	(207)
Kentucky	CTX-M-25/OXA-21 (n=1)	Turkey		Poland/2009	CIP-NAL	PL – A/C	(64)
	SHV-12 (n=3)	Whole chicken and chicken neck skin		Ireland/2008-09	CHL-SUL-TET	PL – NR	(208)

TABLE 3 - *Continued*

***Salmonella* serotype**	**ESBL or AmpC[a] enzyme (no. isolates)**	**Source**	**Concomitant presence in humans[b]**	**Country(ies)/year(s)**	**Non-β-lactam resistance phenotype[c]**	**Genetic element PL – Inc group**	**Reference (s)**
	CMY-2 (n=11)	Chicken meat		Germany/2005	STR	PL – I1	(185)
		Whole chicken		Ireland/2009	(-)	PL – NR	(208)
		Poultry		Canada/1999-2007	(-)	PL – I1	(169)
		Porcine		Canada/1999-2007	(-)	PL – I1	(169)
Kiambu	SHV-2 (n=1)	Abattoir chicken cecum		Canada/2006	STR-SUL-SXT	PL – I1	(197)
Livingstone	SHV-12 (n=9)	Poultry or turkey carcass, poultry faeces, broiler faeces		Italy/2005-06	GEN-NAL-(STR-SUL)	NR	(188)
Llandoff	CTX-M-1 (n=1)	Poultry		France/2006	SUL-TET	PL – I1	(209)
Manhattan	CTX-M-2 (n=2)	Retail Chicken Products		Japan/2000, 2003	(-)	NR	(205)
	CTX-M-15 (n=4)	Retail Chicken Products		Japan/2002, 2003	(-)	NR	(205)
	TEM-52 (n=4)	Retail Chicken Products		Japan/2000, 2002-03	(-)	NR	(205)
	SHV-12 (n=2)	Retail Chicken Products		Japan/2002, 2003	(-)	NR	(205)
	CMY-2 (n=2)	Retail Chicken Products		Japan/2002	(-)	PL – NR	(205)
Minnesota	CTX-M-2 (n=1)	Poultry		Brazil/2009	NR	NR	(184)
	CTX-M-8 (n=1)	Poultry		Brazil/2008	NR	NR	(184)
	CTX-M-14 (n=1)	Poultry		Brazil/2010	NR	NR	(184)
	CMY-2 (n=2)	Poultry		Brazil/2011	NR	NR	(184)
Newport	CTX-M-2 (n=1)	Poultry		Brazil/2008	NR	NR	(184)
Ouakam	CTX-M-1 (n=6)	Turkey		USA/2011	NR	PL – N	(189)
Panama	CTX-M-1 (n=1)	Poultry		Brazil/2009	NR	NR	(184)
Poona	CTX-M-2 (n=1)	Poultry		Brazil/2009	NR	NR	(184)
	CTX-M-8+CTX-M-14 (n=1)	Poultry		Brazil/2005	NR	NR	(184)
Rissen	CTX-M-1 (n=1)	Healthy pigs		Belgium/2009	NR	NR	(170)
	SHV-12 (n=1)	Pig		Spain/1999	STR-SUL-TET	NR	(160)
	CMY-2 (n=1)	Poultry		Brazil/2005	NR	NR	(184)
Saintpaul	CMY-2 (n=1)	Poultry		Brazil/2011	NR	NR	(184)
Schwarzengrund	CTX-M-2 (n=10)	Turkey, chicken, chicken carcass		Brazil/2008	CIP-NAL-STR-TET	PL – P	(183)

TABLE 3 - *Continued*

***Salmonella* serotype**	**ESBL or AmpC[a] enzyme (no. isolates)**	**Source**	**Concomitant presence in humans[b]**	**Country(ies)/year(s)**	**Non-β-lactam resistance phenotype[c]**	**Genetic element PL – Inc group**	**Reference (s)**
Senftenberg	CTX-M-15 (n=1)	Chicken		South Korea/2009	NR	PL – FII	(194)
Typhimurium	CTX-M-1 (n=2)	Poultry meat		Germany/2006	KAN-NEO-STR-SUL-SXT-TET-TMP	PL – I1	(185)
		Swine meat	Yes	Germany/2007	KAN-NEO-STR-SUL-TET-TMP-SXT	PL – N	(185)
	CTX-M-2 (n=10)	Poultry		Brazil/2004-05, 2007	NR	NR	(184)
		Poultry	Yes	Brazil/2003-04	SUL-SXT-TET	PL – NR	(210)
	CTX-M-8 (n=1)	Poultry		Brazil/2011	NR	NR	(184)
	CTX-M-14 (n=1)	Poultry		Brazil/2005	NR	NR	(184)
	CTX-M-15 (n=1)	Broiler		Belgium/2007	(-)	PL – I1	(163)
	TEM-52 (n=6)	Poultry		The Netherlands/2001-02	SPT-SUL-TMP-(NEO-TET)	NR	(187)
		Poultry	Yes	Belgium/2002, France/2004	(-)	PL – I1	(162)
		Healthy pigs		Belgium/2009	NR	NR	(170)
	TEM-52+SHV-12 (n=1)	Poultry meat		The Netherlands/2002	(-)	NR	(211)
	CMY-2 (n=65)	Food from pig		Colombia/UN	OT	NR	(212)
		Pork and pig		Mexico/2002-05	CHL-STR-SUL-TET-(GEN-KAN-NAL-SXT)	NR	(213)
		Retail chicken	Yes	Mexico/2002-04	CHL-STR-SUL-TET-(GEN-KAN-NAL-SXT)	NR	(213)
		Porcine		Canada/1999-2007	CHL-SUL-TET-(GEN)	PL – A/C	(169)
		Swine		USA/2007	CHL-GEN-KAN-STR-SUL-SXT-TET	PL – A/C, FIB, I1	(198)
		Chicken		USA/2007	STR-SUL-TET	PL – A/C, I1	(198)
		Healthy pigs		Belgium/2009	NR	NR	(170)
		Diarrheic pig		South Korea/2011-12	CHL-FFC-GEN-NAL-SXT-TET	PL – A/C, FIB	(171)
Virchow	CTX-M-2 (n=12)	Poultry or poultry product		Belgium/2000-01, 2003	NAL-SUL-TET-TMP-(STR)	PL – HI2	(161)
		Poultry		Ireland/UN	SUL-TET-TMP	PL – P	(190)
		Broiler		Belgium/2001, 2004	STR-SUL-TET-TMP	PL – HI2	(163)
		Poultry	Yes	Belgium-France/2000-03	NAL-SUL-TMP-(STR-TET)	PL – NR	(159)
	CTX-M-2+SHV-2+TEM-1 (n=1)	Broiler		The Netherlands/2002	NAL-SPT-SUL-TET-TMP	NR	(187)
	CTX-M-9 (n=8)	Chicken faeces		France/2002-03	KAN-NAL-SPT-STR-	PL – NR	(158)

TABLE 3 - *Continued*

***Salmonella* serotype**	**ESBL or AmpC[a] enzyme (no. isolates)**	**Source**	**Concomitant presence in humans[b]**	**Country(ies)/year(s)**	**Non-β-lactam resistance phenotype[c]**	**Genetic element PL – Inc group**	**Reference (s)**
					SUL-TET-TMP		
		Chicken faeces and retail chicken meat	Yes	France/2002-03, Spain/2000	NAL-SPT-STR-SUL-TET-TMP-(KAN)	PL – HI2	(161)
		Broiler	Yes	Spain/2000-04	STR-SUL-SXT-NAL-(TET)	NR	(160, 164)
	CTX-M-15 (n=5)	Pigs		Korea/2012-13	GEN-NAL-NEO-STR-TET	HI2	(155)
	CTX-M-32 (n=2)	Poultry		Greece/2001	CHL-KAN-STR-SUL-TET-TMP	PL – NR	(214)
	TEM-52 (n=1)	Poultry		The Netherlands/2002	SPT-STR-SUL-TMP	NR	(187)
Weslaco	CTX-M-8 (n=1)	Poultry		Brazil/2009	NR	NR	(184)
35:c:1,2	SHV-12 (n=5)	Poultry	Yes	Senegal/2000	CHL-GEN-TET-TOB	NR	(215)
4,[5],12:i:-	CTX-M-1 (n=15)	Pig faeces		UK/2009	SUL-(CHL)	PL – I1-γ	(157)
		Swine		Germany/2007, 2009-10	SUL-TET-(STR)	PL – N, I1,	(156)
	CTX-M-2 (n=1)	Avian		Germany/2010	GEN-NAL-SPT-STR-SUL-TET	PL – NT	(156)
	CMY-2 (n=3)	Poultry		Canada/1999-2007	(-)	PL – I1	(169)
		Chicken		USA/2007	KAN-SUL-TET	PL – A/C, N	(198)

Abbreviations: AMK, amikacin; APM, apramycin; AZI, azithromycin; CHL, chloramphenicol; CIP, ciprofloxacin; ENR, enrofloxacin; FFC, florfenicol; GEN, gentamicin; KAN, kanamycin; LVX, levofloxacin; NAL, nalidixic acid; NEO, neomycin; OLA, olaquindox; OT, oxytetracycline; RIF, rifampicin; SPT, spectinomycin; STR, streptomycin; SUL, sulphonamides compound; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; TMP, trimethoprim; TOB, tobramycin; NR, not reported; NT, plasmids that were not typeable with the scheme used; PL, plasmid. Shaded grey lines represent *Salmonella* isolates from pig and/or pork.

[a], Only references with full-characterized ESBL/AmpC genes were considered.

[b], "Yes" – Clones or serotypes simultaneously detected in poultry or pigs, and humans in the same study.

[c], (-), The non-β-lactam resistance phenotype was not detected using the tested antibiotics and the susceptibility criteria adopted. Variable phenotypes were present between curved brackets.

1.2.2.2. Resistance to quinolones in *Salmonella*

Besides the worldwide resistance to clinically important β-lactams, resistance mechanisms that confer decreased susceptibility to fluoroquinolones (see box 6 for details) such as ciprofloxacin, one of the most important antibiotics for treating salmonellosis, have been widely reported in *Salmonella* from humans and food-animals (144, 145).

BOX 6 - Classification and mechanisms of action of quinolones

First generation quinolones (e.g. nalidixic acid) and 2nd to 4th generation fluoroquinolones (e.g. ciprofloxacin, levofloxacin and gemifloxacin, respectively) are bactericidal antibiotics. They inhibit DNA synthesis in bacterial cells by blocking type II topoisomerases (DNA gyrase and topoisomerase IV) at the DNA cleavage stage, preventing strand rejoining and consequently leading to cell death (Figure 9) (144, 147). Type II topoisomerases are tetrameric enzymes, with two A and two B subunits. The A subunits of DNA gyrase and topoisomerase IV are encoded by the *gyrA* and *parC* genes, respectively, while the B subunits are encoded by *gyrB* and *parE* genes, respectively (144).

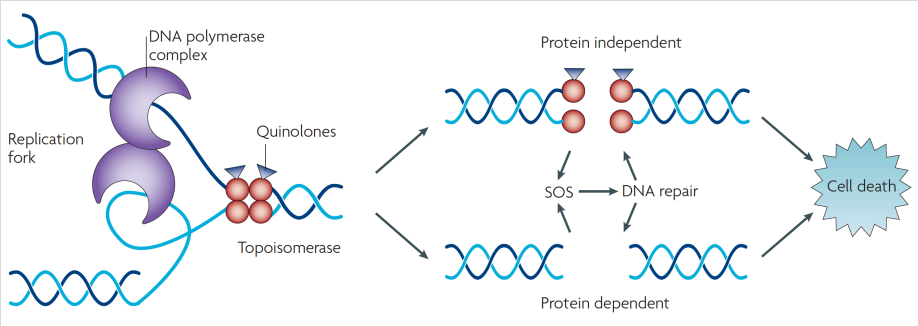


FIGURE 9 – Quinolones target interactions and associated cell death mechanisms [adapted with permission from reference (147)].

In *Salmonella*, the most frequent mechanism of quinolone resistance has been mutations in the quinolone-resistance determining region (QRDR) of *gyrA* (DNA gyrase) and/or *parC* (topoisomerase IV) chromosomal genes (144, 145). Moreover, plasmid-mediated resistance determinants to fluoroquinolones have been described, which typically confer decreased susceptibility to ciprofloxacin, designated plasmid-mediated quinolone resistance (PMQRs)[4]. In both cases, the final level of quinolone resistance displayed is related to the number of resistance mechanisms that are present, either mutations in the organism under consideration and/or the concomitant presence with

[4] The mechanisms of plasmid-mediated quinolone resistance so far described are: i) protection of target enzymes by Qnr proteins blocking their inhibition (e.g. QnrA, QnrB, QnrC, QnrD and QnrS). All the *qnr* gene variants can be accessed online at http://www.lahey.org/qnrstudies/; ii) production of acetylases that affect the activity of some fluoroquinolones and aminoglycosides [e.g. AAC(6')-Ib-cr]; and iii) efflux systems that pump fluoroquinolones out of the bacterial cell (e.g. QepA - major facilitator superfamily and OqxAB - resistance nodulation division family) (144).

additional mechanisms such as PMQRs genes [e.g. *qnr*, *aac(6')-Ib-cr*, *qepA* and *oqxAB* genes] (216).

Among these PMQR mechanisms, Qnr proteins have been commonly described in different clinically relevant *Salmonella* serotypes and geographic locations (Table 4). In fact, in poultry-associated serotypes such as *S.* Enteritidis, S. Heidelberg, *S.* Infantis or *S.* Kentucky several Qnr proteins (e.g. QnrB2, QnrB19, QnrS1) have been frequently reported (Table 4). As can be seen in an European study from Veldman et al (217), poultry seems to be one of the most important vehicles of non-typhoidal *Salmonella* carrying PMQR-*qnr* genes, alerting for the role of food-animals, including animal/food trade, in its dissemination. Nevertheless, an incidence of OqxAB efflux pump and/or the aminoglycoside acetyltransferase AAC(6')-Ib-cr enzymes in pig-associated serotypes such as *S.* Typhimurium and *S.* Derby, recovered from Asiatic pig and pork, have also been recently found (201, 218). In fact, the increasing presence of OqxAB in Asia could be mainly attributed to the widespread use of olaquindox as growth promoter in pig industry (201).

More worrying is that although still rare in *Salmonella* from food-animals, there have been recently some reports displaying an association of PMQR genes with other plasmid-mediated $bla_{\text{CTX-M}}$ genes (144, 219). For example, these associations have been found in IncHI2 plasmids of several MDR *Salmonella* serotypes such as Indiana (*oqxAB*±*aac(6')-Ib-cr*±$bla_{\text{CTX-M-14, -24, -27 or -65}}$) and Enteritidis [*oqxAB*±*aac(6')-Ib-cr*+$bla_{\text{CTX-M-14}}$] mainly recovered from chickens (192, 199-202). These studies highlight the possibility of clinical treatment failure and the additional co-selection potential by two critically important antibiotics, quinolones and β-lactams.

The increasing prevalence of ESBLs, AmpC β-lactamases and/or PMQR genes in MDR *Salmonella* from food-animals and humans is demonstrated as being in a close association with highly adapted clones and/or favoured by horizontal transfer of mobile genetic elements (e.g. plasmids, transposons, genomic islands), two issues that will be addressed in the next topic.

TABLE 4 – *Salmonella* serotypes/clones carrying plasmid-mediated quinolone resistance (PMQR) genes recovered from poultry, pigs and products thereof.

***Salmonella* serotype**	**PMRQ[a] mechanism (no. Isolates)**	**Source[b]**	**Country(ies)/year(s)**	**Antibiotic resistance phenotype[c]**	**Genetic element PL – Inc group**	**Reference(s)**
Agona	QnrB2 (n=1)	Turkeys	Germany/NR	NR	NR	(217)
Braenderup	QnrD (n=2)	Fowls	Spain/NR	NR	NR	(217)
Concord	QnrB2 (n=1)	Pigs	Czech Republic/NR	NR	NR	(217)
Dabou	QnrD (n=1)	Fowls	Spain/NR	NR	NR	(217)
Derby	QnrB2 (n=56)	Fowls or turkeys	Spain/NR	NR	NR	(217)
	QnrS1 (n=1)	Chicken	China/2013	AMP-CHL-CIP-GEN-KAN- NAL-OLA-STR-SUL-TET	NR	(201)
	OqxAB + QnrS8 + QnrB (n=1)	Chicken	China/2013	AMP-CHL-CIP-KAN-NAL-OLA-STR-SUL-TET	NR	(201)
	OqxAB (n=1)	Pork	China/2013	AMP-CHL-CIP-GEN-KAN- NAL-OLA-STR-SUL-TET	NR	(201)
	OqxAB + QnrS1 + QnrB (n=1)	Pork	China/2013	AMP-CHL-CIP-GEN-KAN- NAL-OLA-STR-SUL-TET-(AZI)	NR	(201)
	OqxAB + AAC(6')-Ib-cr + QnrS2 (n=11)	Pork	China/2012-13	AMP-CHL-CIP-GEN-KAN- NAL-OLA-STR-SUL-TET-(AZI)	NR	(201)
	OqxAB + AAC(6')-Ib-cr + QnrS2 + QnrB8 (n=1)	Pork	China/2012	AMP-CHL-CIP-GEN-KAN- NAL-OLA-STR-SUL-TET	NR	(201)
Enteritidis	QnrB2 (n=1)	Fowls or turkeys	Spain/NR	NR	NR	(217)
	QnrB10/B19 (n=1)	Laying hen flock	Poland/2009	CIP-NAL	NR	(64)
	QnrD (n=3)	Fowls	Spain/NR	NR	NR	(217)
	QnrS1/S3 (n=3)	Broiler meat, broiler flock - faeces	Poland/2008-09	AMP-CIP-(STR-TET)	NR	(220)
		Swine flock - faeces	Poland/2008	AMP-CIP	NR	(220)
	OqxAB (n=3)	Chickens	China/2012-13	AMP-CHL-CIF-CIP-ENR-FFC-NAL-OLA-SXT-(GEN-LVX-TET)	PL - HI2, F, N, B/O	(192)
	OqxAB + AAC(6')-Ib-cr (n=1)	Chickens	China/2012-13	AMK-AMP-CHL-CIF-CIP-CTX-ENR-FFC-GEN-LVX-NAL-OLA-	PL - HI2	(192)

TABLE 4 - *Continued*

Salmonella **serotype**	**PMRQ[a] mechanism (no. Isolates)**	**Source[b]**	**Country(ies)/year(s)**	**Antibiotic resistance phenotype[c]**	**Genetic element PL – Inc group**	**Reference(s)**
				SXT-TET		
Give	QnrB19 (n=1)	Imported turkey meat from Brazil	Finland/NR	NR	NR	(217)
Goldcoast	QnrS1 (n=1)	Pigs	Belgium/NR	NR	NR	(217)
Hadar	QnrB2 (n=2)	Fowls or turkeys	Spain/NR	NR	NR	(217)
	QnrB5 (n=4)	Imported turkey meat	Germany/2007	STR-TET	NR	(221)
	QnrB19 (n=15)	Fowls, turkeys	Germany, Denmark/NR	NR	NR	(217)
Havana	QnrB2 (n=3)	Poultry	Portugal/2009-10	(-) or NAL	PL – L/M	(222)
	QnrB19 (n=1)	Poultry	Portugal/2009-10	(-)	PL – HI2	(222)
Heidelberg	QnrB19 (n=2)	Chicken	Venezuela/2005-07, 2008	CIP-GEN	PL – NR	(196)
Indiana	OqxAB (n=52)	Chickens	China/2012-13	AMP-CIF-NAL-SXT-(AMK-CAZ-CHL-CIP-CTX-ENR-FFC-GEN-LVX-OLA-TET)	PL – HI2, F, N, P-PL – 1α, B/O	(192)
		Chicken	China/2008-09	AMK-CHL-FFC-GEN-NAL-OLA-RIF-STR-SXT-(TET)	PL – HI2	(200)
		Duck	China/2009-10	AMP-CAZ-CIP-CRO-CTX-NAL	PL – HI2, N	(199)
	OqxAB + AAC(6')-Ib-cr (n=18)	Chickens	China/2012-13	AMP-CHL-CIF-NAL-SXT-(CIP-CTX-ENR-FFC-GEN-LVX-OLA-TET)	PL – HI2, A/C, N	(192)
		Duck	China/2009-10	AMP-CAZ-CIP-CRO-CTX-NAL	PL – N	(199)
		Chicken	China/2011	AMP-CHL-CIP-GEN-NAL-SXT-TET-(CAZ-CTX)	PL – NR	(202)
		Pig	China/2011	AMP-CAZ-CHL-CIP-CTX-GEN-NAL-SXT-TET	PL – NR	(202)
		Pork	China/2012	AMP-AZI-CHL-CIP-CRO-GEN-KAN- NAL-OLA-STR-SUL-TET	NR	(201)
	AAC(6')-Ib-cr (n=5)	Chicken	China/2011	AMP-CHL-CIP-GEN-NAL-SXT-TET-(CAZ-CTX)	PL – NR	(202)
		Duck	China/2009-10	AMP-CAZ-CIP-CRO-CTX-NAL	PL – N	(199)
	OqxAB + AAC(6')-Ib-cr + QnrB (n=1)	Chicken	China/2012	AMP-AZI-CHL-CIP-CRO-GEN-KAN- NAL-OLA-STR-SUL-TET	NR	(201)

TABLE 4 - *Continued*

Salmonella serotype	PMRQ[a] mechanism (no. Isolates)	Source[b]	Country(ies)/year(s)	Antibiotic resistance phenotype[c]	Genetic element PL – Inc group	Reference(s)
Infantis	QnrS1 (n=1)	Chicken	Germany/2004	AMP	NR	(223)
	QnrB19 (n=1)	Retail chicken	Colombia/2004	KAN-NAL-NEO-STR-TET	PL – ColE like	(224)
Kentucky	QnrS1 (n=1)	Chicken	The Netherlands/NR	NR	PL – N	(225)
London	QnrB2 (n=22)	Fowls or turkeys	Spain/NR	NR	NR	(217)
Mbandaka	QnrB19 (n=1)	Poultry	Portugal/2009-10	NAL	PL – HI2	(222)
Montevideo	QnrB2 (n=3)	Fowls or turkeys	Spain/NR	NR	NR	(217)
	QnrD (n=6)	Fowls, turkeys	Italy/NR	NR	NR	(217)
Newport	QnrB5 (n=3)	Imported turkey meat	Poland/2007	(-)	NR	(221)
	QnrS1/S3 (n=12)	Broiler (meat, flock), turkey meat, goose flock - faeces, duck flock - faeces	Poland/2008-11	CIP-(AMP-CHL-FFC-KAN-STR-SUL-TET-NAL)	NR	(64)
	QnrB19 (n=3)	Turkeys	Denmark/NR	NR	NR	(217)
Ohio	QnrD (n=5)	Fowls	Spain/NR	NR	NR	(217)
Rissen	QnrS1 (n=1)	Pig	Korea/2012-13	NAL	NR	(155)
	OqxAB (n=1)	Pork	China/2013	AMP-CHL-CIP-KAN-NAL-OLA-STR-SUL-TET	NR	(201)
Saintpaul	QnrS1 (n=15)	Imported turkey meat	Germany/2007-08 Poland/2008	STR-TET-(CPD-CHL)	NR	(221)
		Turkeys	Germany, Denmark/NR	NR	NR	(217)
Typhimurium	QnrA1 (n=1)	Turkeys	Germany/NR	NR	NR	(217)
	QnrD (n=2)	Fowls	Spain/NR	NR	NR	(217)
	OqxAB (n=7)	Chicken	China/2007-09	FFC-OLA-SMX-(AMP-CHL-CIF-GEN-NAL-TET)	PL – HI2, F	(218)
		Pigs	China/2010	AMP-CHL-FFC-NAL-OLA-SLX-TET-(GEN)	PL – F	(218)
		Pork	China/2012-13	AMP-CHL-CIP-KAN- NAL-OLA-STR-SUL-TET-(GEN)	NR	(201)

TABLE 4 - *Continued*

Salmonella **serotype**	**PMRQ[a] mechanism (no. Isolates)**	**Source[b]**	**Country(ies)/year(s)**	**Antibiotic resistance phenotype[c]**	**Genetic element PL – Inc group**	**Reference(s)**
	OqxAB + AAC(6')-Ib-cr (n=16)	Chicken, duck	China/2009-10	NAL-OLA-SMX-(AMP-CHL-CIF-ENR-FFC-GEN-TET)	PL – HI2	(218)
		Pigs	China/2008-10	AMP-NAL-OLA-SMX-TET-(CHL-CIF-FFC-GEN)	PL – HI2	(218)
		Pork	China/2012	AMP-CHL-CIP-GEN-KAN- NAL-OLA-STR-SUL-TET	NR	(201)
	OqxAB + AAC(6')-Ib-cr + QnrS1 (n=4)	Pork	China/2012-13	AMP-CHL-CIP-GEN-KAN- NAL-OLA-STR-SUL-TET	NR	(201)
	AAC(6')-Ib-cr + QnrB (n=1)	Chicken	China/2013	AMP-CHL-CIP-GEN-KAN- NAL-OLA-STR-SUL-TET	NR	(201)
	OqxAB + AAC(6')-Ib-cr + QnrS1 (n=1)	Duck	China/2010	AMP-CHL-CIF-FFC-GEN-NAL-OLA-SMX-TET	PL – HI2	(218)
Virchow	QnrS1	Chicken	UK/2004-05	AMP-CIP	N	(226)
		Chicken	Turkey/2005	AMP-NAL	PL – NT	(227)
		Chicken carcass	Korea/2002	AMP-CEF-STR-SUL- TET-TMP	N	(228)
	OqxAB + AAC(6')-Ib-cr + QnrS8 + QnrD (n=1)	Pork	China/2013	AMP-CHL-CIP-GEN-KAN- NAL-OLA-STR-SUL-TET	NR	(201)

Abbreviations: AMC, amoxicillin/clavulanate; AMK, amikacin; AMP, ampicillin; AZI, azithromycin; CAZ, ceftazidime; CEF, cefalotin; CHL, chloramphenicol; CIF, ceftiofur; CIP, ciprofloxacin; CPD, cefpodoxime; CRO, ceftriaxone; CTX, cefotaxime; ENR, enrofloxacin; FFC, florfenicol; GEN, gentamicin; KAN, kanamycin; LVX, levofloxacin; NAL, nalidixic acid; NEO, neomycin; OLA, olaquindox; RIF, rifampicin; STR, streptomycin; SUL, sulphonamides compound; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; TMP, trimethoprim; NR, not reported; NT, plasmids that were not typeable with the scheme used; PL, plasmid. Shaded grey lines represent *Salmonella* isolates from pig and/or pork.

[a], Only references with full-characterized PMQR genes were considered.

[b], No studies were found simultaneously reporting the same *Salmonella* clones or serotypes both in poultry/pigs and humans in the same study.

[c], (-), Antibiotic resistance phenotype was not detected using the tested antibiotics and according to the susceptibility criteria adopted. Variable phenotypes were present between curved brackets.

1.2.3. Acquisition and dissemination of antimicrobial resistance in *Salmonella*

The long-term persistence and dissemination of bacteria in antimicrobial selective environments, including the establishment of well-adapted *Salmonella* serotypes/clones[5], is in part favoured by the vertical (e.g. clonal spread) or horizontal acquisition of genetic units (e.g. plasmids, transposons, integrons, genomic islands) (Figure 10) carrying genes encoding for several adaptive features (e.g. antibiotic resistance and others such as metal/biocide tolerance or virulence) (118). Additionally, the enormous ability of bacteria to undergo different events such as mutations or recombination with foreign DNA, are also major contributors for the evolution of antimicrobial resistant bacterial populations (Figure 10) (118).

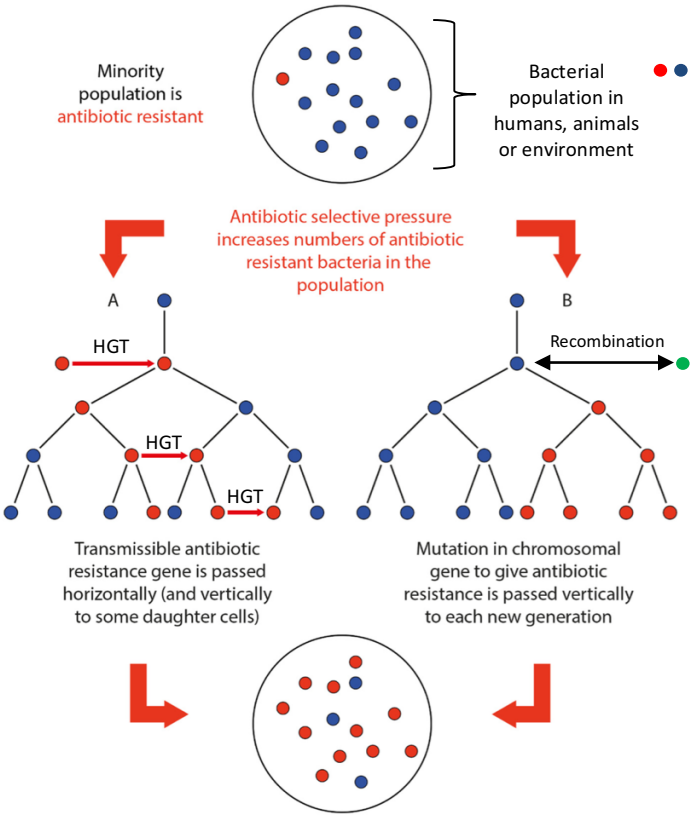


FIGURE 10 – Genetic events responsible for horizontal and vertical transmission of genes that can favour the selection of antibiotic-resistant bacteria. Small filled red circles represent antibiotic-resistant bacteria; Small filled blue circles represent antibiotic-susceptible bacteria; Black lines represent vertical transmission to daughter cells [adapted from reference (119)].

[5] Clone is defined as an isolate or group of isolates descending from a common precursor exhibiting identical or closely similar genotypic traits, which are characterized by a specific strain-typing method (e.g. PFGE or MLST).

1.2.3.1. Clonal spread

The spread of MDR *Salmonella* clones has been often implicated in the dissemination of antibiotic resistance, including of clinically relevant genes encoding for ESBL, AmpC enzymes or PMQR genes, among humans and/or food-animals (120, 137, 144). Those MDR adaptive features might have contributed to their persistence in particular ecological niches and to establish an association to human disease, since several studies provide further evidences of transmission from poultry and pigs to humans of those clinically relevant MDR *Salmonella* clones (17, 120, 137, 144). Interestingly, the high-level resistance to ciprofloxacin (due to *gyrA*/*par*C mutations) and other antibiotics (amoxicillin, streptomycin, spectinomycin, gentamicin, sulfamethoxazole and tetracycline) (18, 62-65) of the previously mentioned *S.* Kentucky ST198-X1-SGI1 strain might also have contributed to its clonal expansion. Additionally, in this particular emerging clone production of carbapenemases (e.g. $bla_{\text{VIM-2}}$, $bla_{\text{OXA-48}}$) and ESBLs (e.g. $bla_{\text{CTX-M-25}}$), has been also reported occasionally (172, 174, 176). In EU, several *S.* Stanley outbreaks linked to turkey meat products consumption have been reported, with the epidemic clone presenting resistance to nalidixic acid and decreased susceptibility to ciprofloxacin (60). Additionally, in the recent outbreak in Austria three strains also presented resistance to gentamicin and cephalosporins (with production of $bla_{\text{CTX-M-15}}$) (60, 61). Several *S.* Infantis successful clones of broiler origin are also characterized by MDR profiles, including nalidixic acid, tetracyclines, sulphonamides or nitrofurans (50, 57, 113), antibiotics frequently used in livestock production, which could contribute to their co-selection in the poultry industry and consequently for its transmission to humans. In Belgium and France, particular MDR clones of *S.* Infantis carrying also $bla_{\text{TEM-52}}$ gene have been found among poultry and human origins (162).

Concerning pig-associated serotypes, antimicrobial resistance acquisition to agents commonly used in livestock production (e.g. tetracycline's, sulphonamides) has also been associated with the spread of particular *Salmonella* serotypes/clones in both food-animals and humans. One example is the dissemination of the epidemic *S.* Typhimurium DT104 clone carrying the typical MDR ACSSuT (A-amoxicillin, C-chloramphenicol, S-streptomycin, Su-sulfamethoxazole and T-tetracycline) profile (17, 91, 107), in some cases associated with ciprofloxacin and/or extended-spectrum cephalosporin resistance (e.g. $bla_{\text{TEM-52}}$) (162). More recently in Europe the emergence and persistence of the most important *S.* 4,[5],12:i:- clones have been also related to diverse MDR profiles, particularly the European clone with ASSuT and the Spanish clone with ACGSSuTTm (G-gentamycin, Tm-trimethoprim) (89, 229). In addition, the description of ESBL genes ($bla_{\text{CTX-M-1}}$) in *S.* 4,[5],12:i:- isolates from pig sources belonging to the successful European clone is of

concern, due to the possibility of dissemination of these clinically relevant genes (156). Recently, the high incidence of PMQR determinants OqxAB ± Aac(6′)-Ib-cr that has been observed in Asia, appears to be associated with the clonal spread of a MDR *S.* Typhimurium, initially reported in food-animals (218) and later in human isolates (230, 231).

1.2.3.2. Horizontal gene transfer

Several studies have demonstrated that antimicrobial resistance determinants can be mobilized inside or out of the bacterial cells, most likely by intracellular (mobile genetic elements – MGE – such as transposons, gene cassettes and insertion sequences that can move from one genetic location to another in the same cell) or intercellular mobility (plasmids, transposons, genomic islands that can move from one bacteria to another) (148, 232, 233). Intercellular movement of DNA can occur via conjugation (transfers of genetic material from donor to recipient's cells through bacterial pilus), transformation (uptake of free DNA) or transduction (via bacteriophages) (Figure 11) (232).

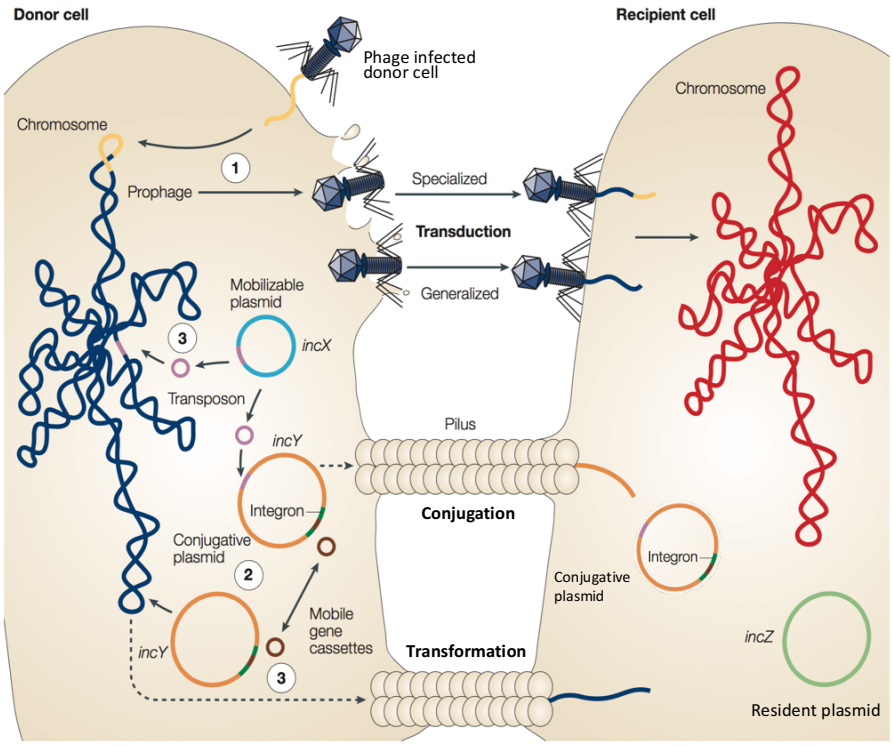


FIGURE 11 – Horizontal gene transfer of antimicrobial resistance: (1) – transduction, (2) – conjugation and (3) - transposition [adapted with permission from reference (232)].

In *Salmonella* the major conjugative or mobilizable genetic elements that have contributed to its evolution, particularly with regard to the acquisition and dissemination of antimicrobial resistance, were plasmids, genomic islands, transposons and integrons/gene cassettes (234). Plasmids[6] are the elements considered as the main drivers responsible for the dissemination of antibiotic resistance, including PMQRs, ESBLs and AmpC enzymes, in Gram-negative bacteria, inclusive in *Salmonella* (234-236). These genetic units are typically composed of conserved backbone modules with genes encoding for replication, maintenance and transfer functions as well as variable accessory modules (e.g. ABR, biocides tolerance), which mediate bacterial adaptation (148, 234). They are classified according to different criteria, such as the number of copies, host range (broad or narrow), their ability to transfer (conjugative or mobilizable) between cells and the incompatibility group (Inc) (i.e. two plasmids belonging to the same Inc can not coexist in the same cell) (148, 232). Incompatibility groups have been defined for plasmids of *Enterobacteriaceae*, using a PCR-based replicon-typing scheme, targeting sequences encoding replication (237). In *Salmonella*, transmission of clinically relevant genes have been associated with diverse Inc plasmids, such as the narrow host IncI1 (e.g. $bla_{\text{CTX-M-1}}$, $bla_{\text{TEM-52}}$ and $bla_{\text{CMY-2}}$) and IncHI2 (e.g. $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-9}}$, *oqxAB* and/or *aac(6')-Ib-cr*) or the broad host IncA/C (e.g. $bla_{\text{CMY-2}}$) and IncN (e.g. $bla_{\text{CTX-M-15}}$), in both human and food-animals suggesting a direct link between the two bacterial populations trough food-chain (Table 3 and 4) (161, 162, 168, 193, 204). One recent example was the presence of the same clone of *S.* Infantis carrying a $bla_{\text{CTX-M-1}}$-conjugative megaplasmid with other antibiotic resistance genes (~ 280–320Kb), isolated from Italian broiler chickens, broiler meat and humans, suggesting a clonal *Salmonella* transmission from the broiler chicken industry along the food-chain (204). Also the same clone of *S.* Enteritidis harbouring a FIIs plasmid with CTX-M-15 and several ABR genes was found in chicken, chicken meat and human stool (193). *S.* Heidelberg clones carrying I1 plasmids with the AmpC-$bla_{\text{CMY-2}}$ enzyme were also found in both human and food-animal samples (e.g. chicken, turkey) (168).

Besides plasmid encoding antimicrobial resistance genes, a number of genomic islands[7] (GIs) (238, 239), including *Salmonella* GI 1 variants (SGI1) have been associated with the spread of antibiotic resistance mainly to ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracycline (240). One of the best well-known examples is the worldwide dissemination of the epidemic strain *S.* Typhimurium DT104

[6] Plasmids are usually circular, self-replicating DNA molecules that normally exist in cells as extra-chromosomal replicons.
[7] Genomic islands (GIs) are large chromosomal regions, usually carrying mobility genes coding for integrases or transposases that are required for chromosomal integration and excision, but also other additional genes encoding for adaptive features. These elements are part of the flexible gene pool, being frequently associated with tRNA genes and flanked by direct repeat structures and insertion sequences (238, 239).

from both humans and from food-producing animals (240). These isolates carried a mobilizable SGI1 (241, 242), consisting of a backbone with a class 1 integrons (In104 of the In4 type) that includes a pentaresistant phenotype [*aadA2-bla*$_{PSE-1}$-*sul1-tet(G)-floR-cmlA1*] (242). In fact, integrons[8] (243), mainly class 1 integrons in *Salmonella*, seems to be an important source of acquisition of antibiotic resistance genes (91). The presence of a 2000bp integron carrying *bla*$_{OXA-30}$ and *aadA1* gene cassettes in a MDR *S.* Typhimurium clones was also identified in both animal and human isolates (91, 244).

It is known that successful MDR *Salmonella* serotypes/clones persistence and spread among food-animals and the environment is derived, in part, to the acquisition of resistance mechanisms to commonly used antibiotics in animals and humans. However, several entities, pointed out tolerance to other non-antibiotic compounds with antimicrobial activity (e.g. metals, biocides), widely used in food-animals as a potential adaptive feature to different environments and hosts (animal/human) (245). More worrying is that persistence of these metals/biocides in different environments can contribute for the selection of metal/biocide tolerant and/or antibiotic-resistant bacteria due to mechanisms of cross and co-resistance (245, 246) (more details in the next topics).

1.3. Bacterial tolerance to biocides, particularly to metals

As discussed in the previous topics, antibiotics are considered one of the biggest selective pressures in diverse ecological niches for the emergence of antibiotic-resistant bacteria (118). With the ban of antibiotics as growth promoter's in the EU (247), the use of other non-antibiotic compounds with antimicrobial activity (e.g. metals/biocides) are currently applied in the food-animal setting to control and reduce foodborne zoonotic pathogenic bacteria (245, 248). Recently, the European Commission highlighted that these broad use of metals/biocides (even in subinhibitory concentrations) might contribute to the selection and maintenance of antibiotic-resistant bacteria in some ecological niches (245, 248). In fact, the activity of antibiotics and metals/biocides is often diminished by the same bacterial mechanism (cross-resistance) and/or by different mechanisms codified by genes co-located in common mobile genetic platforms (co-resistance), such as plasmids, genomic islands and transposons (245, 246, 249). These important issues will be addressed in the next topics.

[8] Integrons are assembly platforms that incorporate exogenous open reading frames by site-specific recombination and convert them to functional genes by ensuring their correct expression. They are composed of three key elements: a gene encoding an integrase (*intI*), a primary recombination site (*attI*), and a promoter (P_c) that directs the transcription of the captured genes. At present, five classes of integrons (defined based on the sequence of the encoded integrases) are known, all physically linked to mobile DNA elements, being class 1 integrons the most widespread in *Enterobacteriaceae* (243).

1.3.1. Definition, classification, application and accumulation of metals and biocides

Biocides are chemical compounds capable of killing or inhibiting microorganisms (250). According to the Directive no. 98/8/EC (251), biocidal products are defined as "active substances and preparations containing one or more active substances, put up in the form in which they are supplied to the user, intended to destroy, deter, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism by chemical or biological means". They are classified into at least 22 types of biocidal products distributed by 4 main groups (i.e. disinfectants, preservatives, pest control and others) (252). Additional classifications for biocides may be used based either on the functional group it possesses (e.g. alcohols, aldehydes, metals, halogens, quaternary ammonium compounds - QACs) or the bacterial target on which they act (e.g. proteins, membrane, nucleic acids or cell wall) (Table 5) (250).

TABLE 5 – Classification of biocides based on the bacterial target [adapted from reference (250)].

Membrane	Proteins	Nucleic acids	Cell wall
• QACs • Biguanides • Phenols • Phenylethers • Acids • Terpenes • Alcohols • Anilides • Peroxygens • Parabens • Isothiazolones • Anionic surfactant	• Alcohols • Phenols • Phenylethers • Aldehydes • Metals derivatives • Isothiazolones • Acids (parabens) • Peroxygens • Chlorine compounds • Biguanides • Vapor-phase disinfectant	• Alcohols • Acids (parabens) • Antimicrobial dyes • Acridines • Biguanides • Aldehydes • Diamidines • Chlorine compounds • Metals derivatives • Peroxygens • Halogens • Vapor-phase disinfectant	• Alcohols • Phenols • Aldehydes • Chlorine releasing compounds • Metals derivatives (mercurials)

Contrarily to antibiotics, which activity is frequently systemic, biocides are mainly applied as disinfectants, antiseptics or used as preservatives in a variety of settings (245, 253). Those included for example the hospital setting to control nosocomial infections (e.g. disinfectants on medical devices and surfaces, antiseptics used on skin and mucosa), in the food industry (e.g. preservation of food, surface disinfectants) and in the consumer's home within several products (e.g. cosmetics and personal care products, household products and textiles) (245, 253, 254). Additionally, in the food-animal setting biocides also played a key role for controlling microorganisms in animal farming management (biosecurity, hygiene), due to the mandatory need for limiting the presence of pathogens, such as *Salmonella*, in the food supply (29, 255-258). In fact, these

compounds (e.g. chlorhexidine, halogens, silver, copper, QACs, triclosan) have been largely applied for avoiding animal colonization/infection and contamination across the food chain (245, 259), from farms (including in animals) and hatcheries through slaughter facilities and dairy processing plants to food processing, packaging and retail facilities (Figure 12) (245, 253, 254, 259, 260). Additionally, metals can also be used in agriculture as pesticides and in soil fertilizers (e.g. arsenic) (246, 261) and can be applied as antimicrobial coatings and in animal wound dressings (e.g. silver, copper and zinc) (260, 262, 263). Also important is the extensive use of copper and zinc in feed or water (feed additive) as micronutrients and/or for growth promotion of certain farm animals and for enteric disease control (246, 264-267). In Europe, zinc has been usually incorporated in feed at concentrations not exceeding 250 mg/kg, for all animal species (264, 268). Zinc in USA is used between 50-100 ppm for dietary requirements, although higher concentrations can be used for growth promotion (2000-3000 ppm) (269). In the same way, copper has been used in feed at different concentrations according to animal type, ranging from a maximum of 175 mg/kg (~2.8 mM) in piglets to a minimum of 35 mg/kg (~0.6 mM) in species or animal categories other than piglets and ovines (264, 270). In USA copper supplements are not restricted and the typical amounts range from 125 ppm to 250 ppm (~2 to 4 mM) in all animal's diets (271). Other metals, such as arsenic, are still used as growth promoter in some countries (e.g. China) in the form of roxarsone, but in others (e.g. USA) occurred a voluntary withdrawn (261).

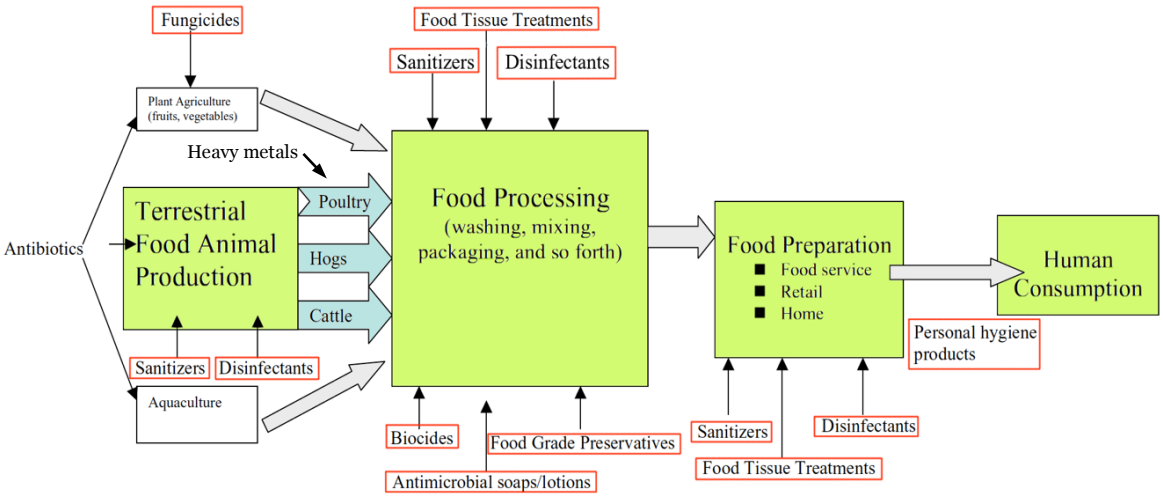


FIGURE 12 – Main sites of application of biocides in the food-animal setting as part of biosecurity measures [adapted with permission from reference (272)].

As a consequence of the extensive application of metals, including in feed for food-animals, they can be environmental pollutants and accumulate for example in manure or

waste lagoons in the animal production setting (246, 266, 273), and then be spread by sewage sludge and liquid manure applied in amended soils worldwide or by water bodies (246, 274-276). In fact, high concentrations (e.g. up to 3387.6 mg/kg, mg/kg; 53 mM) of copper have been observed in pig manure and consequently pig manure/sludge-amended soils (275-279). Other metals such as mercury and arsenic, considered as food and/or feed contaminants (273, 280) and tellurium, a contaminant mainly due to industrial pollution (281), could also contribute for these global burden of environmental metal contamination.

In contrast to the monitoring use of antibiotics in human and animal health (e.g. DANMAP[9]-Denmark; EMA/ESAC[10]-Europa; FDA[11]-USA), there are few free publicly available compilations of data on sales or consumption of various metals and biocides (245). According to the last report of the European Commission, in 2009 the estimated annual total volume of sales of biocidal active ingredients (production and imports) in the EU was about 400.000 tonnes, most of which belonged to the class of disinfectants, with a greater incidence on the use of silver salts/nanoparticles (282, 283). In fact, production volumes of many of these compounds are considered to be several orders of magnitude higher than those of antibiotics (245).

Given its importance in the food-animal setting, in the next topics a focus will be given to the metals copper and silver widely applied in the animal welfare and, additionally, to three environmental, food and/or feed contaminants, tellurium, mercury and arsenic.

1.3.2. Mechanisms of action of the biocide activity of metals

Based on studies of the mechanisms of action of antimicrobial agents it is well known that antibiotics act in specific bacterial cell targets, resulting in the inhibition of essential physiological processes (e.g. nucleic acid synthesis, folate biosynthesis) or alteration of cellular structures (146, 147). Contrarily, biocides at in use-concentrations (i.e. concentrations, usually high, of the biocidal product applied in a real context) may have multiple and unspecific target sites within the cell (e.g. interaction with outer cellular components, cytoplasmic membrane, proteins, DNA, RNA and/or other cytosolic components), acting through physicochemical interactions or chemical reactions (Figure 13) (250, 260, 284, 285). For example, the main antimicrobial mechanism of action of

[9] DANMAP is the Danish Programme for surveillance of antimicrobial consumption and resistance in bacteria from animals, food and humans.
[10] EMA is the European Medicines Agency; ESAC is the European Surveillance of Antimicrobial Consumption Network.
[11] FDA is the United States Food and Drug Administration.

QACs (e.g. benzalkonium chloride, cetrimide) is primarily related to their cationic surfactant (detergent) properties (286). In fact, the association of QACs at in-use concentrations, with the phospholipids on the bacterial cytoplasmic membrane, leads to physical disruption and partial solubilisation of membrane, allowing vital intracellular components to be released (250, 253, 286, 287).

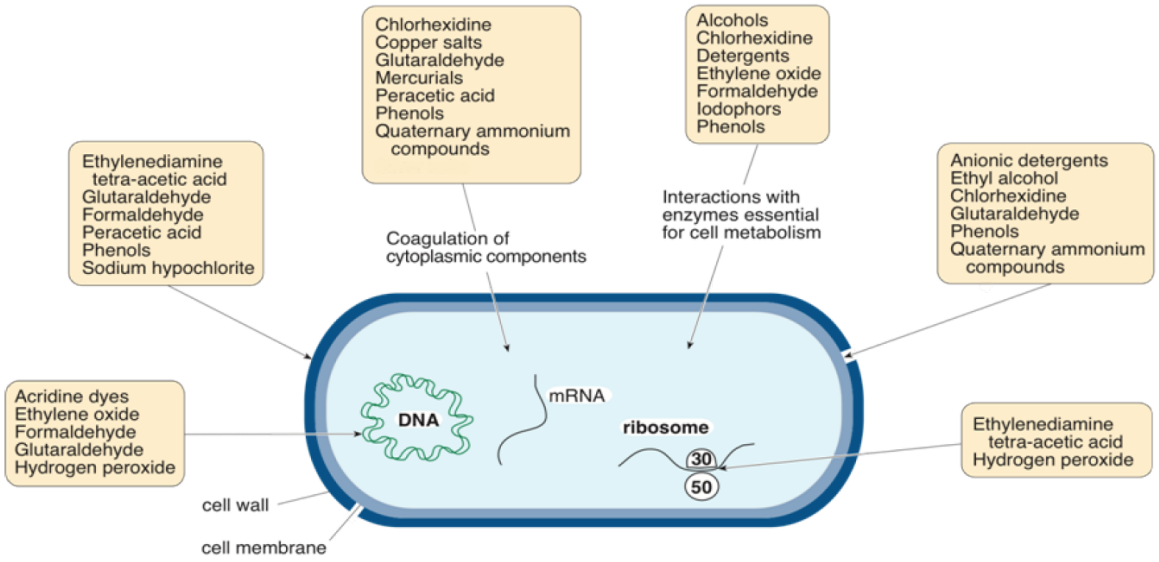


FIGURE 13 – Mechanisms of action and bacterial targets of biocides [reprinted from reference (259)].

Until now, several mechanisms of toxicity in bacteria have been described for metals (Figure 14), such as formation of reactive oxygen species and antioxidant depletion [e.g. As(III), Cu(II), Te(IV)], protein dysfunction [e.g. Ag(I), Cu(I), Hg(II), Te(IV)], impaired membrane function [e.g. Ag(I), Cu(II)] (e.g. compromising the cytoplasmic membrane integrity, disruption the activity of the bacterial electron transport chain) or genotoxicity [e.g. As(III), some organomercury complexes] (262, 263, 288, 289). Concerning some of non-essential metals for the cell, such as silver (Ag), mercury (Hg) or tellurium (Te), it is known that they are extremely toxic to most bacteria, having microbicide activity even at very low concentrations (288).

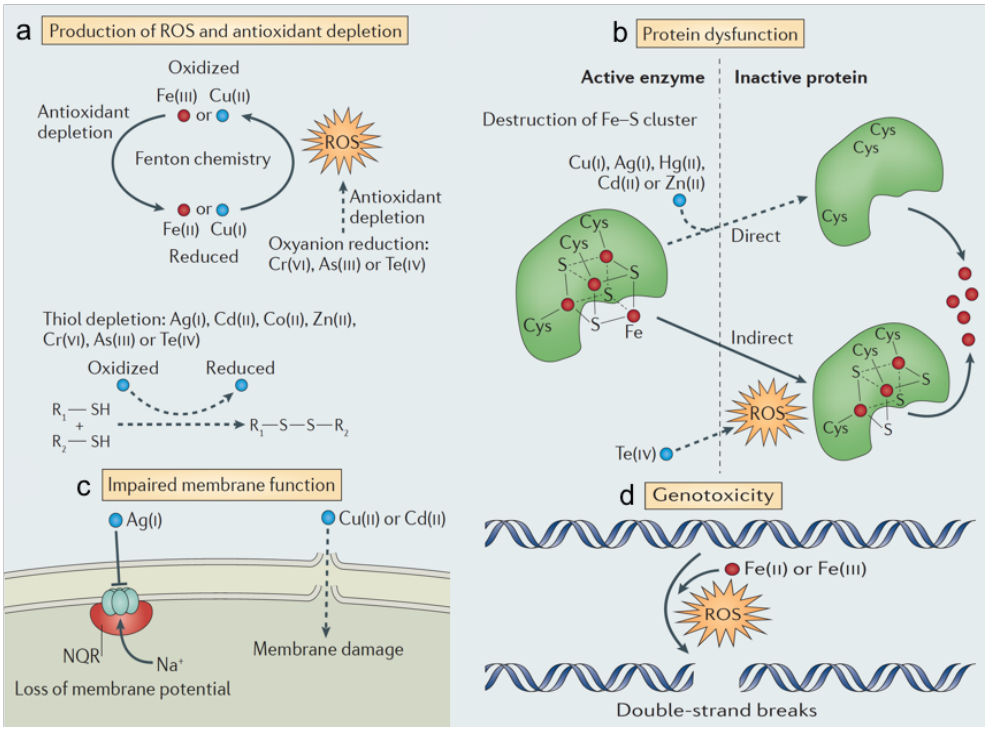


FIGURE 14 – Antimicrobial mechanisms of metal toxicity; Solid arrows represent pathways in which the underlying biochemistry has been elucidated, whereas dashed arrows represent a route of toxicity for which the underlying biochemical mechanism is unclear; ROS - reactive oxygen species [adapted with permission from reference (288)].

Contrarily, metals such as copper (Cu), are essential for eukaryotic and prokaryotic cellular functions (290). In fact, the essentiality of Cu, lies in its capacity to undergo redox cycling between the oxidized cupric form Cu^{2+} (borderline Lewis acid) and, the more toxic reduced cuprous form Cu^{+} (soft Lewis acid) (291, 292). Because of this property, the Cu^{+}/Cu^{2+} couple with a high redox potential can act as electron donor/acceptor of many cupro-proteins (291, 292). Many of these enzymes are essential for diverse processes, such as oxidative phosphorylation (e.g. enzyme cytochrome *c* oxidase), iron homeostasis (e.g. ceruloplasmin) or superoxide dismutation (e.g. superoxide dismutase) (290, 292, 293). Nevertheless, at toxic concentrations, Cu can damage bacterial cells by multifactorial mechanisms of action (Figure 14). In fact, an important role for copper in phagosomal killing of bacteria has been reported, especially for *Salmonella*, a pathogen with an intracellular lifecycle, since during copper deficiency this ability seems to be diminished (Figure 15) (294, 295). Under aerobic condition, Cu can take part in Fenton-like reactions leading to oxidative damage by the generation of reactive oxygen species (ROS) (e.g. hydroxyl radicals, hydrogen peroxide and superoxide), which in turn damage lipids, proteins and DNA (263, 295). In the absence of oxygen, disruption of iron sulphur clusters in dehydratases involved in processes such as

branched-chain amino acid synthesis seems to be the most important mechanism (291, 295, 296).

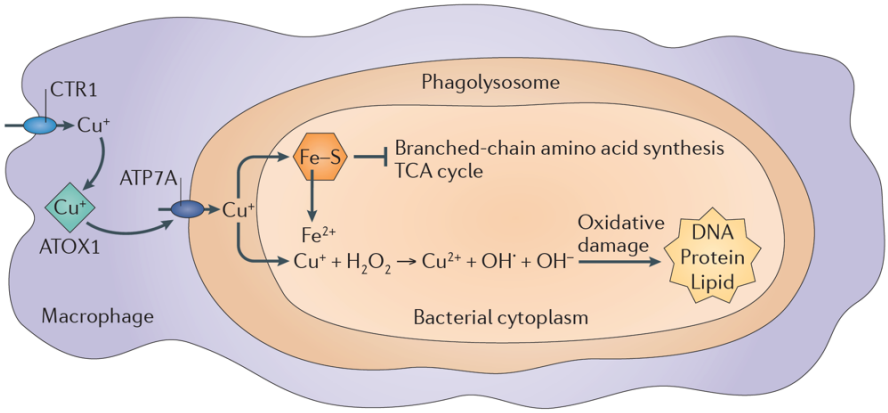


FIGURE 15 – Copper mechanisms of action within macrophages of mammalian host, following phagocytosis of bacteria. CTR1 is a Cu^+ transport protein; ATOX1 is an antioxidant 1 copper metallochaperone and ATP7A is a copper-transporting P-type ATPase [reprinted with permission from reference (295)].

Apart from the several mechanisms of metal/biocide toxicity previously described, there are a number of intrinsic[12] (e.g. concentration, contact time and stability of the biocide) and extrinsic[13] (e.g. temperature, pH, presence of organic matter mainly at the food-animal setting, presence of biofilms or microorganisms that are able to inactivate the biocide molecule) factors that could have a great influence in the efficacy of a biocide (297). Consequently, depending on the biocidal concentrations reached in a particular environment, the growth, metabolism/physiology and division cycle of the bacterial cell may be affected at different extents, which might contribute to the selection of tolerant bacteria (298). Tolerance mechanisms of metals included in the experimental work of this thesis will be discussed in the next topics.

1.3.3. Mechanisms of tolerance to metals, particularly in *Salmonella*

In the field of metals/biocides, it is known that terms such as "resistance" and "tolerance" have acquired recently specific technical meanings, although they have been

[12] The impact of intrinsic factors on the biocide activity depends on the biocide compound specific chemical features and the way it is applied by the user.
[13] The impact of extrinsic factors on the biocide activity depends on the environmental conditions that are present during application of biocide.

applied rather loosely in several studies. The term bacterial resistance should be used when the phenomenon being studied is bacterial cell killing (297). According to Maillard and colleague's (299) biocide resistance is considered when a microbicide is ineffective against a microorganism that was previously susceptible to that microbicide. Contrarily, the term bacterial "tolerance" should be used while discussing adaptation to inhibitory concentrations of the biocide compared to wild type populations (297). The tolerance of a microorganism to a biocide comparing to the wild type populations might occur to concentrations well below those used in products applied in hospitals, domestic and industrial practice. Thus, these more tolerant microorganisms apparently do not compromise the biocide effectiveness in daily applications (297, 299). As in this thesis studies we evaluated the phenotypic changes, usually through the determination of MIC values compared to wild type bacterial populations henceforth we will use the term "tolerance".

Unlike antibiotics, a major problem when assessing tolerance to this type of compounds is the lack of available standard methodologies, which in turn leads to poor levels of comparability between studies and a lack of reliable data (245, 299). Indeed, in the case of antibiotics, standardized test methods allowed to define clinical breakpoints and epidemiological cut-off values (ECOFFs) based in MICs (134), which in the case of metals/biocides are currently scarce (300, 301). Development of standardized techniques is currently important for application in metal/biocide tolerance surveillance programmes, for providing informative data in biocidal product development or usage, and for helping to establish regulatory policies.

To persist and survive in metal contaminated environments, bacteria have evolved mechanisms to handle with toxic concentrations of these compounds (263). Metal tolerance in bacteria is not usually limited to a single mechanism but rather a combination of them, since these compounds have multiple targets inside the cell (288). There are several mechanisms of tolerance that bacteria use to withstand metal toxicity, such as, reduced uptake (e.g. regulation of transporters involved in metal uptake), efflux (e.g. membrane transporters, driven by ATP hydrolysis or chemiosmotic potential, able to transport specific toxic metals out of the cell), extracellular sequestration (e.g. up regulation of the expression of extracellular polymers or siderophores), intracellular sequestration (e.g. metal precipitation or use of cytoplasmic proteins, such as bacterioferritin and metallothioneins), repair, metabolic by-pass (e.g. production of alternative proteins with modified catalytic cores) or chemical modification (e.g. reactions that alter the chemical reactivity of the metal atoms) (Figure 16) (263, 288). Additionally,

physiological adapted bacterial communities such as in biofilms, were shown to withstand metal toxicity (302) as also previously demonstrated for antibiotics.

Some of these metal tolerance genes/mechanisms, also present in *Salmonella*, are centralized in an online platform (http://bacmet.biomedicine.gu.se/) (303) and will be detailed in the next topics.

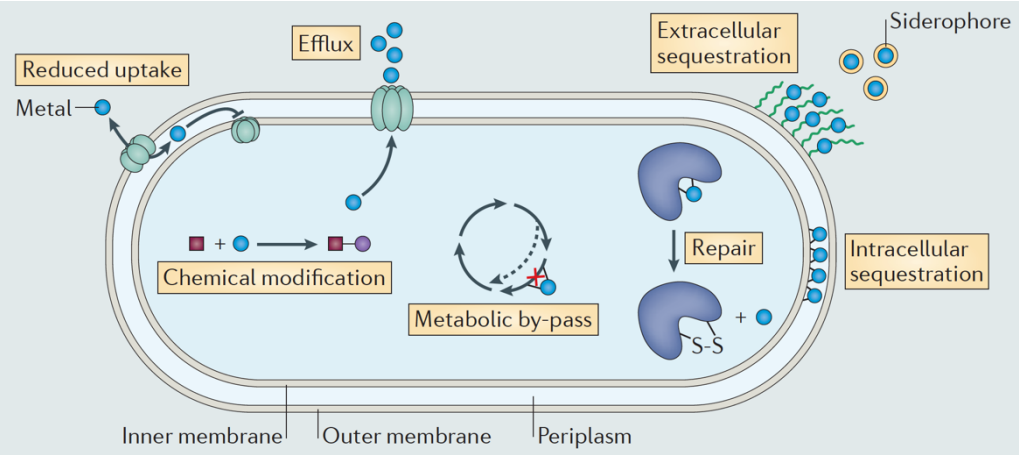


FIGURE 16 – Intrinsic or acquired metal tolerance mechanisms [adapted with permission from reference (288)].

1.3.3.1. Copper homeostasis and tolerance mechanisms

Copper is an essential metal required in many biological processes (292). However, toxic Cu concentrations for microbes can occur during cellular immunological or predation strategies (e.g. macrophages, amoebas) and anthropogenic activities (e.g. animal feed additives, agriculture microbicides) in diverse environments (e.g. manure, soil) (246, 292, 304). To cope with both the essentiality and toxicity of Cu, *Enterobacteriaceae*, including *Salmonella*, have evolved by elaborating interwoven mechanisms of tolerance, homeostasis and delivery pathways, which act to tightly control intracellular Cu pools (305). In general, these mechanisms include Cu efflux (e.g. chromosomally or plasmid encoded efflux systems), Cu sequestration (e.g. by chaperones or siderophores) and copper oxidation (e.g. mixed copper oxidases and superoxide dismutase mimics) (293).

In *Salmonella*, intracellular copper homeostasis both in aerobic and anaerobic conditions is maintained mainly as the result of the activity of Cue (Cu efflux) system (306, 307). This system consists of the copper sensing MerR-family transcriptional regulator CueR that mediates copper-responsive expression of the cytoplasmic PIB1-type ATPase Cu^{+} efflux protein CopA, the periplasmic multi-copper oxidase CuiD (re-oxidation of Cu^{+} to Cu^{2}; also involved in *Salmonella* virulence) (308) and the periplasmic copper binding protein CueP (chaperone; particularly relevant in anaerobic conditions) (Figure

17) (294, 305-307, 309, 310). Besides the Cue system, the cytoplasmic copper-handling regulatory circuit is partially duplicated in *Salmonella* (305). In fact, an additional Gol system has been identified, consisting of a second CueR-like sensor GolS (primarily associated with gold sensing) and a cytoplasmic Atx1/CopZ copper chaperone-like protein GolB (311, 312). Also, a PIB1-type ATPase GolT, that together with CopA are involved in efflux as part of a Cu detoxification and a supply route to the periplasmic superoxide dismutase SodCII by the CueP chaperone, is also present (Figure 17) (305). Since *Salmonella* does not have the CusCBA HME-RND[14] type efflux system, such as *E.* coli to transport Cu from the periplasm to outside the cell, a TolC protein was recently proposed as a potential outer membrane copper exporter in aerobic conditions (313).

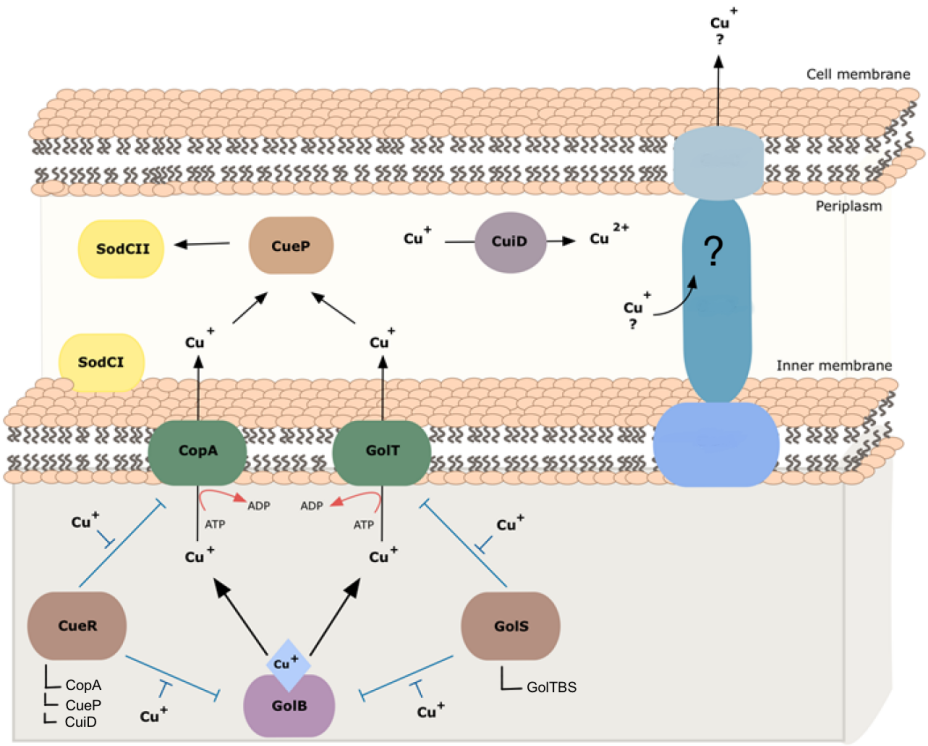


FIGURE 17 – Copper homeostasis mechanisms based in Cue and Gol systems in *Salmonella* [adapted from reference (314)].

In environments where copper concentrations would overcome chromosomally encoded copper homeostatic systems, bacteria would be able to tolerate these higher copper values if they contain additional plasmid-encoded copper tolerance mechanisms (293). These extrachromosomal tolerance systems are usually metal oxidation state selective (293). The best know Cu tolerance mechanism although not yet full characterized is the *pcoABCDRSE* cluster (Figure 18). This cluster was firstly described in a *E. coli*

[14] HME-RND is a Heavy Metal Efflux Resistance-Nodulation-Division system.

pRJ1004 plasmid (with MIC_{CuSO_4} up to 12-20 mM) recovered from a piggery effluent (315), and later in other *Enterobacteriaceae* such as *Salmonella* (with MIC_{CuSO_4} up to 18 mM) (316).

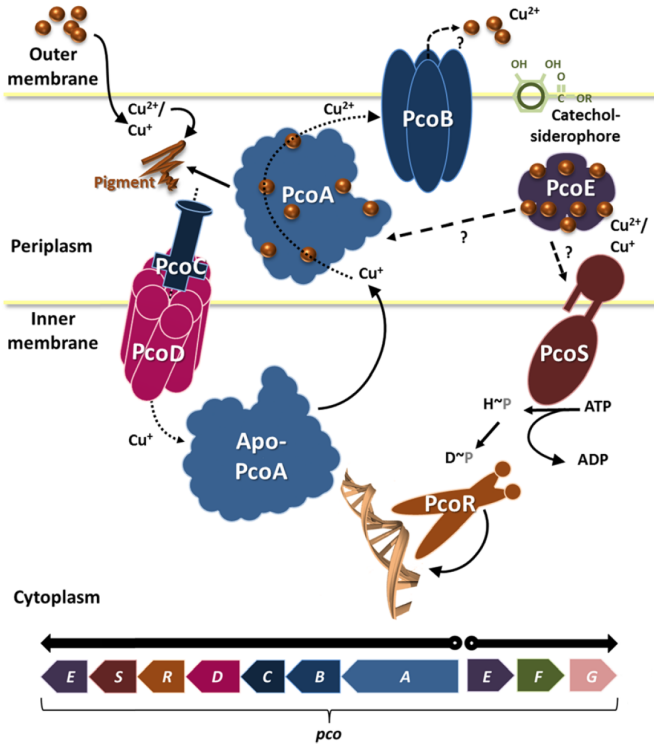


FIGURE 18 – Proposed copper (*pco* cluster) tolerance mechanism. The bottom line indicates the genes and their transcriptional and translational directions, with the open circles representing potential promoter regions/transcript start sites [adapted with permission from reference (304)].

The transcription of PcoABCD proteins appears to be regulated by PcoRS and cusRS seems to regulate PcoE protein (263, 304, 317). PcoA is a multi-copper oxidase and may have a similar function to CueO, oxidizing Cu^{+} to less toxic Cu^{2+}. PcoB possibly functions as the outer membrane transporter (263, 304, 317). Sitting in the inner membrane, PcoD drives the transport of Cu^{+} from the periplasm to the cytoplasm, with periplasmic PcoC chaperoning/delivering the Cu^{+} to PcoD (263, 304, 317). PcoE is an additional chaperone that binds Cu^{+}/Cu^{2+} but also Ag^{+} and in the periplasm and probably shuttles it to PcoA and/or PcoS (317). The roles of PcoFG have not been elucidated. This *pco* cluster (only *pcoA* searched) has already been detected in a collection of specific *Salmonella* isolates from pig and related environments (318). However, the MIC_{CuSO_4} (16-24 mM) values detected were higher in aerobic conditions both in isolates carrying *pco* or not (318), which indicate that these genes might not lead to an increase in copper tolerance in such environmental conditions. Although similar MIC_{CuSO_4} values (20-28 mM) were previously found in *Salmonella* isolates from pig farms, in this case no

genotyping studies were performed to detect the presence of *pco* cluster (319). More recently, Hao and colleagues (304) have discussed the possibility of *pco* gene cluster be part of a genetic cluster comprising other metal tolerance determinants, such as the *sil* gene cluster associated with Ag tolerance. These authors proposed that those gene clusters might be a possible "copper pathogenicity island", associated or not with Tn*7*-like elements (263, 304, 320-322).

1.3.3.2. Silver tolerance mechanisms

Silver is a non-essential metal, being extremely toxic to most bacteria, thus some tolerance mechanisms to overcome high concentrations of Ag have been described. The first described, and to date best-characterized Ag^+ tolerance mechanism, is the *silCFBAGPRSE* gene cluster. It was firstly described in a 180kb IncHI2 plasmid, pMG101, of a *S.* Typhimurium from a burn unit (323). The region that is responsible for Ag tolerance (MIC_{AgNO3}>256 mg/L; >1.5 mM), the inducible *silCFBAGPRSE*, comprises 9 genes (Figure 19) (304). In fact, 8 of these genes were primarily characterized based on homologies with other known metal tolerance determinants, such as the *cus* system of *E. coli* (324).

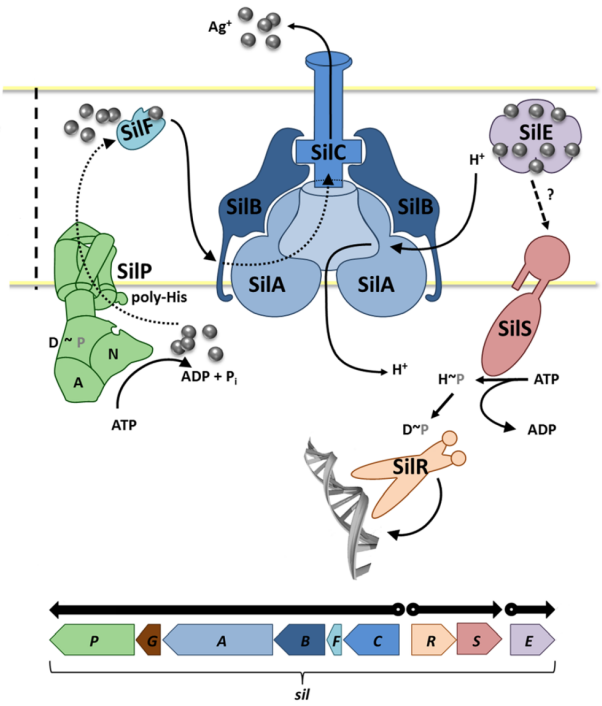


FIGURE 19 – Proposed silver (*sil* cluster) tolerance mechanism. The bottom line indicates the genes and their transcriptional and translational directions, with the open circles representing potential promoter regions/transcript start sites [adapted with permission from reference (304)].

SilE is predicted to bind Ag^{+} and Cu^{2+} in aerobic conditions (325), transporting them to the HME-RND efflux system (SilCBA), responsible for exporting the metal ions out of the cell. Additionally, it was also proposed that this *silE* gene may have an additional role in Cu tolerance under more reducing environments (e.g. anaerobic conditions found in animal gut/manure/waste lagoons) by binding to the more toxic form Cu^{+} (260, 304, 325). SilF is predicted to act as a metal chaperone to SilCBA too. The other putative efflux pump present in *sil* cluster is SilP, a P-type cation ATPase, which transports Ag^{+} ions from the cytoplasm to the periplasm (262, 323, 326). While the expression of *silCFBAP* is thought to be controlled by the two component membrane sensor and transcriptional responder SilRS, the expression of *silE* is thought to be regulated/co-regulated by the Cus system (304, 317).

The *sil* genes have been reported as scattered in different bacterial genera, although most studies have been focused in non-*Salmonella Enterobacteriaceae* isolates (e.g. *Enterobacter cloacae* and *E. coli*), particularly recovered from nosocomial and animal/human clinical settings (325, 327-330) or environmental bacteria (326). The *sil* gene cluster, alone or associated with *pco* or other metal tolerance genes, has also been identified in *E. coli* pAPEC-02-R (331), *Serratia marcescens* pR478 (332), *Klebsiella pneumonia* pK2044 and pLVPK plasmids (333, 334). In *Salmonella*, *sil* associated with *pco* cluster has been found in sequenced chromosomes and plasmids of some serotypes (304). Although silver tolerance genes have been already reported, an association between the presence of the *sil* cluster and the corresponding phenotypic behaviour, obtained by different susceptibility methods, has only been established in few *Enterobacteriaceae* isolates (327-330, 335), remaining unexplored in comprehensive *Salmonella* collections.

1.3.3.3. Mercuric ion tolerance mechanisms

Mercury (Hg) is toxic to humans and with no known positive role in cellular function. However, it has been widely used in chemical, agriculture and pharmaceutical industry (e.g. thimerosal, merbromin, nitromersol) since the XIX century (336). Nowadays, due to its toxicity, the usage of antimicrobial mercury compounds is in decline and is likely to be replaced (263). Elemental Hg^{0} (non-toxic) present in the air is able to settle into the water or land, oxidized to Hg^{2+} and transformed into the highly toxic form of methyl mercury (336). This cycle regularly exposes microorganisms, humans, animals and plants to different Hg forms (inorganic and organic), by different routes (e.g. water, soil, food chain) (337).

Bacterial tolerance to Hg, including in *Salmonella*, is mainly due to the presence of the *mer* operon, that comprise a group of proteins involved in the detection, scavenging,

transport and reduction of Hg (336, 338). Transcription of *mer* operon is induced (or repressed) by the product of *merR* gene in the presence (or absence) of Hg^{2+} ion, and its expression is further regulated by *merD* (339). Two types of *mer* operons were mainly described including those conferring tolerance only to inorganic Hg salts (narrow type) or both to organomercurials and inorganic Hg salts (broad type) (336, 338, 339). All types of *mer* operons contain a gene encoding a mercuric reductase (MerA) responsible for the enzymatic reduction in the cytosol of Hg^{2+} to the volatile Hg^{0} (Figure 20) (336, 339). The periplasmic Hg^{2+} scavenging protein (MerP) and one or more inner membrane spanning proteins (MerT, MerC, MerE, MerF, MerG) that transport Hg^{2+} to the cytoplasm are also reported as part of the *mer* operon (Figure 20) (336, 339). Broad types of *mer* operons also contain a organomercurial lyase MerB that hydrolyses organic radicals before the action of *merA* (339).

Most of *mer* genes are described to be widespread among diverse bacterial hosts, genetic platforms and environments (339, 340). Among *Salmonella*, *mer* operons or *merA* gene were recently found downstream of the chromosomal RR1–RR2 antibiotic resistance region in *S.* 4,[5],12:i:- (229), similar to those reported in several *Salmonella* genomic island 1 (SGI1) variants (240, 341, 342). Additionally, this operon was found in transposons, particularly of the Tn*21*-like family (340), widespread in different *Enterobacteriaceae,* including in the recent *E.* coli O104:H4 mass food poisoning outbreak isolates from 2011 (343, 344) and in *Salmonella* isolates from diverse serotypes (321, 341, 342). *Salmonella* conjugative plasmids of IncA/C, IncF and IncHI2 families from human and food-animal samples (e.g. chicken, pig, cattle) carrying *mer* genes have also been reported (182, 321, 345-353). In fact, a recent study demonstrated that a *S.* Infantis clone associated with several outbreaks, carrying a plasmid harbouring the *mer* operon was able to grow in the presence of 25 mM mercury compared to the pre-emergent strain (without the *mer* operon), highlighting the role of *mer* operon for Hg tolerance in contaminated environments (113).

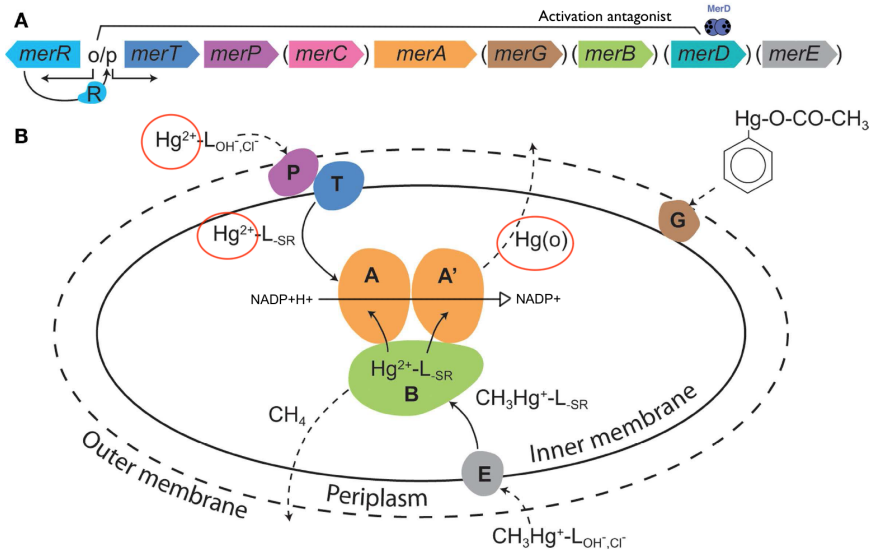


FIGURE 20 – Proposed mercury (*mer* operon) tolerance mechanism; a) the generic *mer* operon with curve brackets representing variable genes, b) The cellular *mer*-encoded mercury detoxification mechanisms, L=ligand with subscripts denoting the ligand type [adapted from reference (339)].

1.3.3.4. Tolerance mechanisms to metalloids arsenic and tellurite

During several years' organic arsenic (As) derived compounds, such as carbarsone, nitarsone and roxarsone have been used as feed additives for poultry, in the USA and China, acting as growth promoters and in controlling coccilobacillosis disease (263, 354). A recent study suggested that organoarsenic feed additives in the animal wastes are an important source of arsenate and arsenite pollution in Chinese fertilized agricultural lands, where organoarsenic compounds are still widely used (355). Only very recently, in June 2011, the US Food and Drug Administration announced the voluntary withdraw of the sale of roxarsone due to the presence of highly toxic inorganic arsenic residues in chicken meat from chickens fed on roxarsone-supplemented feeds (354). Arsenics can be found in animals and plants combined with carbon and oxygen to form organic arsenic compounds or in the environment combined with oxygen, chlorine or sulphur to form highly toxic inorganic As compounds [e.g reduced form As(III) (arsenite) and oxidised As(V) (arsenate)] (263, 356).

In *Enterobacteriaceae*, including *Salmonella*, the most common As tolerance mechanism, usually conferring tolerance to As(III), As(V) and antimonial compounds, is associated with the presence of a 5-gene system *ars* operon usually plasmid located (Figure 21) (356). Although the minimum *ars* operon consist always of *arsR* [As(III)-

responsive *trans*-acting transcriptional repressor protein], *arsB* [$As(OH)_3/H^+$ antiporter protein that could be an ATP-dependent process or require the help of ArsA] and *arsC* [arsenate reductase that reduces As(V) to As(III)], additional genes such as *arsA* [ATPase that provides energy to ArsB for the efflux of As(III), which is more efficient than ArsB alone] and *arsD* [encoding a negative regulatory protein that binds to the promoter region of the operon and represses the transcription but also a metallochaperone for As(III) efflux via ArsAB] could also be present (Figure 21) (263, 356, 357).

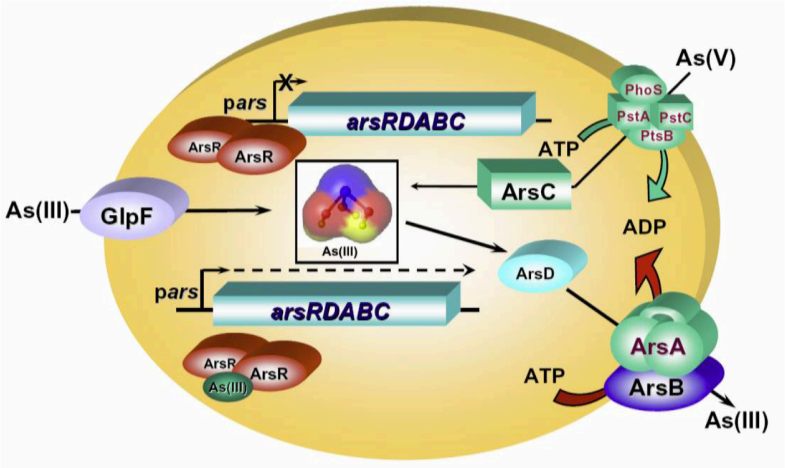


FIGURE 21 – Proposed arsenic (*ars* operon) tolerance mechanism. Arsenic enters the cell as either As(III) by the aquaglyceroporin, GlpF or As(V) by *pst* and *pho* phosphate permeases [reprinted from reference (357)].

In *Salmonella*, arsenic tolerance mechanisms are less described than in other *Enterobacteriaceae*, such as *Klebsiella pneumoniae* carrying $IncFII_K$ plasmids with *ars* genes (e.g. GenBank accession nos. CP000648, JX430448, CP002474, KF719971) (358, 359). However, these genes have already been reported in *Salmonella* chromosome (360) as well as in few plasmids from IncH (161, 348) and IncI1 families (361). In addition, very few studies in *Enterobacteriaceae*, including *Salmonella*, have demonstrated an association between the *ars* operon and arsenic tolerance and, when available, the majority of susceptibility techniques performed are quite different between studies, hindering results comparison. Joerger and colleagues (360) have demonstrated a higher incidence (in all studied isolates) of *arsB* and *arsD* genes in *S.* Kentucky, a USA poultry-associated serotype, comparing with other *Salmonella* serotypes (26/58 positive strains). In this study, with few exceptions, the acquisition of *ars* genes was correlated with a certain level of arsenate tolerance [ars^+= 0.5-10 mM *versus* $ars^-\leq$ 0.1 mM As(V)] and arsenite [ars^+= 0.1-1 mM *versus* $ars^-\leq$ 0.05 mM As(III)], highlighting the role of *ars*

operon for survival in arsenic contaminated environments (e.g. animal cecum or litter sources) (360).

Tellurite [TeO_3^{2-} ion or Te(IV)], is the oxyanion of the chalcogen tellurium (Te), that can be reduced to the insoluble and less toxic Te^0 (362, 363). Tellurite is highly toxic to bacteria (concentrations as low as 1 mg/mL) and therefore, was used for many years as an antimicrobial and therapeutic agent in medicine (364). Currently several forms of tellurium are used in industry (e.g. cadmium telluride solar panels, steel manufacturing industry, pesticide in agriculture) and as a selective agent in diverse microbiological culture media for the isolation of pathogens (362, 364). Until now six bacterial tellurite tolerance mechanisms have been proposed (coded by *ter*, *teh*, *tel*, *tpm*, *cysK*, and *ars* genes), although the genetic, biochemical and/or physiological bases underlying their tolerance have not been defined (363). Apparently, tellurite-tolerant bacteria can be associated with mechanisms that cause oxyanion extrusion or biochemical modifications different from reduction (e.g. generation of methylated forms of Te) (362). One of the most important mechanisms in *Enterobacteriaceae*, and that usually confers higher levels of TeO_3^{2-} tolerance (512-1024 μg K_2TeO_3/ml), is that encoded by the *ter* operon (common *terZABCDE* ± *terF* or *terW*) (363). Currently little is known about the function of *ter* operon genes, including the tolerance mechanism that remains unknown (363).

In *Salmonella* this *ter* operon has been frequently associated with *ars* operon, the two ubiquitously present in IncH plasmids (161, 182, 321, 348). This determinant was also found in IncH (e.g. GenBank accession nos. AP006726, BX664015, DQ517526, JX182975) and IncF (e.g. GenBank accession nos. CP001919) plasmids from other *Enterobacteriaceae*, being also associated with pathogenicity islands (e.g. tellurite genomic islands-TER-GIs) of *Escherichia coli* O157:H7 (365, 366), and with hyper virulent clonal groups of *Klebsiella pneumonia* (367).

There is no doubt that metals and biocides are invaluable compounds that provide society with a number of benefits, contributing for the general human and animal health. However, the widespread use of these compounds, including in the food-animal setting, led to the emergence of some concerns by the European Union Scientific Committee for New and Emerging Health Risks, including the emergence of metal and biocide tolerance and their potential role for the selection and persistence of antibiotic-resistant bacteria (discussed in the next topic).

1.3.4. Bacterial tolerance to metals and biocides and the possible link with the selection and persistence of antibiotic-resistant *Salmonella*

The serious concern that exposure of bacteria to metals/biocides may boost the spread of antibiotic resistance has contributed to the establishment of a new European Biocidal Product Regulation (252). This new document requires that biocidal products manufacturers, besides the evaluation of safety and efficacy of the substances, ensure that they will not contribute to antimicrobial resistance development (252). Additionally, extensive metals/biocides environmental contamination, even at low-level concentrations, is a topic of increasing concern recognized by diverse authorities and authors (246, 265, 266, 270, 368, 369). Since some of those compounds, including metals, may not be completely degraded, they can persist in the environment, contributing for the selection of metal tolerant and/or antibiotic-resistant bacteria by mechanisms of cross or co-resistance (246, 249, 267). Metals can also potentially participate as catalysts of gene transfer among diverse microorganisms sharing the same ecologic niche (246, 369). In fact, recent environmental studies in metal contaminated soils/animal manure, particularly with Cu due to anthropogenic-derived sources (e.g. animal feed additives, agriculture microbicides), have shown that metal availability (e.g. 22.4-3172 mg/Kg; ~0.4-50 mM of Cu), even at low level concentrations, was correlated with a high prevalence of antibiotic resistance in diverse bacteria (277-279, 370, 371). Although the previous studies have shown the prevalence of antibiotic-resistant bacteria in the presence of metals/biocides, the factors behind the selection and maintenance of these antimicrobial resistant bacteria were not fully elucidated.

In *Salmonella* tested in laboratory conditions, exposure of *S.* Typhimurium to different concentrations of biocides (e.g. QACs, triclosan, oxidizing compound, aldehyde, oil phenol) was able to select for mutants with decreased biocide susceptibility, with most of them also often displaying decreased susceptibility to various antibiotics, mainly by overexpression of AcrAB-TolC efflux pump, indicating that biocides can mediate co-selection of antibiotic resistance by cross-resistance mechanisms (372-375). As previously mentioned co-selection of antibiotic-resistant bacteria can be due to cross-resistance, which describes one mechanism that counteracts two or more antimicrobial agents, such as antibiotics and metals/biocides (249). Probably one of the most important mechanisms in *Enterobacteriaceae*, including *Salmonella*, is related to the wide spectrum of substrates

(e.g. QACs[15], antibiotics-fluoroquinolones) recognized by different chromosomally or plasmid encoded efflux systems (Figure 22) (253). In fact, overexpression of *Salmonella* and *E. coli* AcrAB-TolC efflux pumps is hypothesized to mediate cross-resistance to fluoroquinolones, a clinically important antibiotic, and QACs (253, 376, 377). Additionally, the transferable plasmid-encoded RND multidrug efflux pump, OqxAB, recently dispersed in *S.* Typhimurium (201, 218), showed to confer reduced susceptibility to biocides-QACs (benzalkonium chloride and to some extent cetrimide) and to diverse antibiotics (fluoroquinolones, chloramphenicol, trimethoprim and olaquindox) (378), which is of special concern due to the current use of fluoroquinolones for treating invasive salmonellosis.

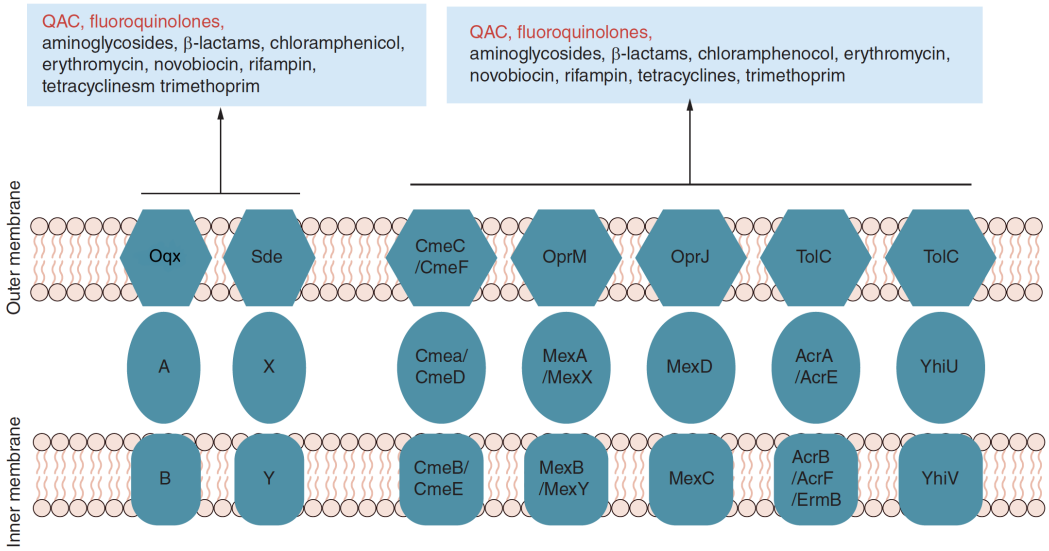


FIGURE 22 – Representation of the main types of multidrug-resistance efflux pumps involved in extrusion of QACs and several other antibiotics in Gram-negative bacteria [reprinted from reference (253)].

Besides cross-resistance, probably one of the most important co-selection mechanisms in *Enterobacteriaceae* is co-resistance (246, 249). This can result from the acquisition of two or more resistance/tolerance genes linked together in the same genetic unit (such as a plasmid, transposon, genomic island or integron) (246, 249). This physical linkage results in the co-selection of all the genes in the same element even in the presence of a single environmental selective pressure (e.g. antibiotics or metals/biocides) (246, 249). In fact, Pal and colleagues (379) have recently demonstrated the co-occurrence of

[15] The primary mechanisms of QACs tolerance in Gram-negative bacteria, including *Salmonella*, are due to the acquisition of genes for specialized QAC efflux pumps or overexpression of those chromosomally encoded systems (e.g. AcrAB-TolC proteins from RND family) (287). Although, other mechanisms such as reduced expression of porins (e.g. S. Typhimurium exposed to sub lethal concentrations of three QACs, where found with reduced levels of the outer membrane porins OmpC, OmpF and OmpA) could also be present (374).

metals/biocides tolerance and antibiotic resistance genes in fully sequenced bacterial genomes and plasmids from several bacteria taxa. These authors have found metals/biocides tolerance genes in 86% of bacterial genomes, being 17% co-located with antibiotic resistance genes, although these datasets are not representative of the full microbial diversity (379). Independently of the chromosomal or plasmid location, bacteria with metals/biocides tolerance genes carried more frequently antibiotic resistance genes (379). Additionally, the metals/biocides tolerance genes commonly co-occurring with antibiotic resistance genes were mercury tolerance and the *qacEΔ1* genes (379). This data is not surprising since for example class 1 integrons, widely distributed in *Enterobacteriaceae*, including *Salmonella* from human and food-animal origins, are usually associated with *qacEΔ1* genes plus sulphonamide resistance (*sul1* ou *sul3*) and other antibiotic resistance gene cassettes (e.g. trimethoprim - *dfrA1* and *dfrA12* and aminoglycosides - *aadA2*) (91, 380-382). In fact, it has been described that class 1 integrons are more prevalent in bacteria exposed to detergents/biocides and that these compounds could co-select for antibiotic resistance due to the co-location of both genes in the same genetic elements (383). Similarly, the widespread of mercury resistance genes has been associated with Tn*21* family, with most of these transposons also carrying class 1 integrons and antibiotic resistance genes (263, 340)). In *Salmonella*, *mer* operons have been found increasingly associated with multidrug resistance genes, either associated with chromosomal resistance region (ASSuT phenotypes) as in the case of *S.* 4,[5],12:i:- European clone (229) or with SGI1 variants (e.g. SGI1-K1 AGSSuT profile; SGI1-K2 ASSuT; SGI1-L1 ACGSSuTTr) in diverse *Salmonella* serotypes (240, 341, 342, 384). Although widely reported in *Salmonella*, a correlation between environmental mercury exposure and increased antibiotic resistance has never been established for this foodborne pathogen.

Pal et al (379) also found that clinically important genera mainly recovered from human and domestic animals, including *Salmonella*, tended to carry both metal/biocide tolerance and antibiotic resistance genes on the same plasmids. Notably, the frequency of antibiotic resistance genes was significantly higher on metal/biocide tolerance genes-carrying plasmids, than plasmids without metal/biocide tolerance genes (379). This is not uncommon since a number of plasmids from different families (e.g. IncH, IncA/C), also present in *Salmonella*, have been reported as carrying both metals (*sil*/*pco* clusters, *mer*, *ars*, *ter* operons) and multiple antibiotic resistance genes (304, 320, 321, 349). In fact, recently, an *in vitro* assay showed that the presence of sub lethal levels of Cu or As were sufficient to co-select for the maintenance of *E. coli* and *Klebsiella pneumoniae* MDR plasmids (385), highlighting the role of these compounds for selection and persistence of bacteria carrying these genetic elements. Also, Cavaco and colleagues (386, 387) argued

that the use of zinc in feed could be an additional factor that may have contributed to the emergence of Meticillin-Resistant *Staphylococcus aureus* (MRSA), although in this case the antibiotic resistance and metal tolerance genes did not have a plasmid location. These authors have found that several MRSA strains carried *czrC* gene, associated with reduced zinc and cadmium susceptibility, in the SCC*mec* genomic island characteristic of these strains either from human or food-animals (386-388).

Some of the previously reported evidences highlight that a variety of biocides and metals (e.g. widely used as disinfectants, antiseptics/medicines, preservatives, growth promoters) found in the food-animal setting and other diverse polluted environments could have the ability to co-select for antibiotic-resistant bacteria and/or resistance plasmids (246, 249). Nevertheless, a comprehensive analysis of metal tolerance, including acquired metal gene dispersion and their genetic context within different *Enterobacteriaceae*, including *Salmonella*, from diverse ecological niches or belonging to frequent or emergent MDR clones, remains poorly explored. Additionally, we urgently need to be aware of the simultaneously action of biocides/metals with other stressors (e.g. antibiotics; reducing environments) for an enhanced survival, selection and persistence of particular metal/biocide tolerant-MDR strains.

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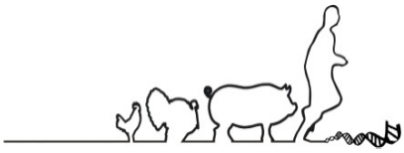


Chapter 2

Objectives and outline of the study

"Imagination is more important than knowledge."

Albert Einstein

2.1. Statement of objectives

Non-typhoidal *Salmonella enterica* constitutes a worldwide major foodborne zoonotic pathogen. Its main reservoir is the intestinal tract of food-producing animals, which readily leads to contamination of farm surrounding environments and the food chain.

In recent years, expansion of particularly successful *Salmonella* serotypes and clones have been reported globally. They are transmitted to humans mostly by a wide range of foodstuffs of animal origin, they are involved in endemic/epidemic situations and they frequently show resistance to multiple antibiotics limiting therapeutic options when necessary. Interventions related to the limitation of antibiotics use (e.g. growth promoters ban in EU) were unsuccessful to control antibiotic resistance expansion, revealing the need for new approaches. In fact, recent priority programs included the study of the factors promoting antibiotic resistance in animal-environment-human interface (http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_028.pdf; http://www.onehealthinitiative.com/about.php), and stressed the need to overcome several knowledge gaps. Among them are included the effects of non-antibiotic compounds with antimicrobial activity, such as metals/biocides (incorporated in disinfectants, antiseptics, preservatives and as feed additives) in the selection and evolution of antibiotic resistant bacteria, namely in food-animal production environments.

The currently available information concerning the emergence and persistence of successful MDR *Salmonella* serotypes/clones in different environments and hosts is mostly related to the acquisition and maintenance of genetic elements harbouring antibiotic resistance genes. Indeed, most of these studies have been focused in the selection pressure created by the massive use of antibiotics, other than the human applications widely present in different food-animal production settings. More recently, diverse acquired genes coding for metals/biocides tolerance mechanisms have been described in scarce studies of *Salmonella* and other Gram-negative bacteria, but they only covered a few isolates/serotypes, sources and did not used a phenotypic/genotypic multilayered approach. As so, a comprehensive analysis of metals/biocides tolerance, including their acquired gene dispersion and genetic contexts within *Salmonella* from diverse ecological niches or belonging to successful or emergent MDR clones, remains poorly explored.

Our ***hypothesis*** was that tolerance to metals/biocides contributes for the survival and emergence/persistence of particular multidrug-resistant (MDR) *Salmonella* serotypes and clones in diverse environments and hosts.

The general ***goal*** of this work was to assess the occurrence of acquired metals/biocides tolerance genes as well as to characterize their associated genetic platforms and tolerance phenotypes in different *Salmonella* serotypes and emergent clones.

To accomplish this purpose, 406 isolates from a collection of ~2500 Portuguese non-typhoidal *Salmonella enterica*, representative of different serotypes and clones (years 2000 to 2014) were included. They were selected based on the source (human clinical cases, food products, food-animal production setting and the aquatic environment), geographical regions (North, Centre, South, Islands) and antibiotic susceptibility phenotypes and genotypes, Pulsed-Field Gel Electrophoresis (PFGE) types and/or Sequence Types (ST; obtained by Multilocus Sequence Typing-MLST).

This study focused on the following ***specific aims***:

1. *To analyse the occurrence and dispersion of genes associated with tolerance to copper, silver, mercury, arsenic and tellurite*. We intended to determine whether *Salmonella* isolates from different ecological communities and hosts, are important reservoirs of metals/biocides tolerance genes.

2. *To understand the epidemiological and genetic backgrounds of Salmonella isolates carrying metals/biocides tolerance genes*. We intended to verify if the acquisition of metals/biocides tolerance was more associated with frequent or emergent MDR *Salmonella* serotypes and/or clones involved in human infections and if this feature could be an additional advantage for their maintenance in different ecological niches and hosts.

3. *To assess the susceptibility of diverse Salmonella serotypes/clones with different genetic backgrounds to copper and silver, two commonly used metals in food-animal setting*. We expected to unveil the advantage conferred by the acquisition of copper/silver tolerance genes to overcome metal toxic concentrations occurring in different food-animal settings, which might facilitate particular serotypes/clones survival and persistence in these environments. The phenotypic assays under

anaerobic atmospheres besides the aerobic classical ones intended to mimic oxygen conditions in diverse copper contaminated environments and hosts (e.g. manure, animal gut, waste lagoons) and to evaluate if anaerobic environments might have an important role in the selection of metal tolerant *Salmonella*.

4. *To characterize the genetic platforms involved in the acquisition of metal/biocide tolerance and to assess their transfer ability along with antibiotic resistance.* We intended to assess if metals/biocides tolerance genes were co-located in the same genetic platforms as antibiotic resistance genes, able to be co-selected by different compounds present in the food-animal setting. A detailed analysis of *Salmonella* available genomes was also performed in order to evaluate if predominant genetic platforms associated with several metals/biocides tolerance genes are spread in different *Salmonella* serotypes/clones recovered from different sources.

2.2. Outline of the thesis

The findings that would answer the *specific aims* of the thesis yielded different publications (n=6; 5 publications in peer review journals and 1 manuscript under final revision). Such results have been organized throughout the papers, according to the rationale proposed as follows:

I. *Insights on the population structure* and antimicrobial resistance *of clinically-relevant Salmonella* serotypes *from clinical and non-clinical settings*. In addition to isolates of food and human origin previously characterized in studies performed by our group, these works further characterized *Salmonella* serotypes with human clinical relevance recovered from pigs farms (e.g. *S.* Rissen, *S.* Typhimurium) and also belonging to the worldwide emergent serotype, *S.* 4,[5],12:i:-. The introduction of these pig-related serotypes was crucial since metals, such as copper and silver, are currently widely used in pig-production as animal feed for growth promotion and as disinfectants/antiseptics, respectively.

a. Antunes P, **Mourão J**, Pestana N, Peixe L. 2011. Leakage of emerging clinically relevant multidrug-resistant *Salmonella* clones from pig farms. *Journal of Antimicrobial Chemotherapy*. 66(9):2028-2032.

b. **Mourão J**, Machado J, Novais C, Antunes P, Peixe L. 2014. Characterization of the emerging clinically-relevant multidrug-resistant *Salmonella enterica* serotype

4,[5],12:i:- (monophasic variant of *S.* Typhimurium) clones. *European Journal of Clinical Microbiology & Infectious Diseases*. 33(12):2249-57.

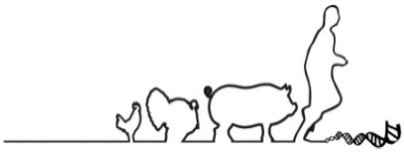
II. *Analysis of the dispersion of metals/biocides tolerance genes and tolerance phenotype as well as their associated genetic platforms in diverse non-typhoidal Salmonella serotypes and clones.*

c. **Mourão J**, Novais C, Machado J, Peixe L, Antunes P. 2015. Metal tolerance in emerging clinically relevant multidrug-resistant *Salmonella enterica* serotype 4,[5],12:i:– clones circulating in Europe. *International Journal of Antimicrobial Agents*. 45(6):610-6.

d. **Mourão J**, Marçal S, Ramos P, Machado J, Peixe L, Novais C, Antunes P. 2015. Tolerance to multiple metal stressors in emerging non-typhoidal MDR *Salmonella* serotypes: a relevant role for copper in anaerobic conditions. *Submitted to the Journal of Antimicrobial Chemotherapy (under the second editorial revision).*

e. Antunes P, **Mourão J**, Alves T, Campos J, Novais C, Novais A, Peixe L. 2013. *Salmonella enterica* serotype Bovismorbificans, a new host for CTX-M-9. *International Journal of Antimicrobial Agents*. 41(1):91-93.

f. Campos J, **Mourão J**, Marçal S, Machado J, Novais C, Peixe L, Antunes P. 2015. Clinical *Salmonella* Typhimurium ST34 with metal tolerance genes and an IncHI2 plasmid carrying *oqxAB-aac(6')-Ib-cr* from Europe. *Journal of Antimicrobial Chemotherapy*. DOI: http://dx.doi.org/10.1093/jac/dkv409.



Chapter 3

Results and discussion

"You can never solve a problem on the same level on which it was created."

Albert Einstein

3.1. Insights on the population structure and antimicrobial resistance of clinically-relevant *Salmonella* serotypes from clinical and non-clinical settings

3.1.1. Leakage of emerging clinically relevant multidrug-resistant *Salmonella* clones from pig farms.

3.1.2. Characterization of the emerging clinically-relevant multidrug-resistant *Salmonella enterica* serotype 4,[5],12:i:- (monophasic variant of *S.* Typhimurium) clones.

Leakage of emerging clinically relevant multidrug-resistant *Salmonella* clones from pig farms

Patrícia Antunes[1, 2], Joana Mourão[2], Nazaré Pestana[2] and Luísa Peixe[1]*

[1] Faculdade de Ciências da Nutrição e Alimentação, Universidade do Porto, Rua Dr. Roberto Frias, 4200 Porto, Portugal; [2] REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, no. 164, 4050-047 Porto, Portugal.

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Leakage of emerging clinically relevant multidrug-resistant *Salmonella* clones from pig farms

Patrícia Antunes[1,2], Joana Mourão[2], Nazaré Pestana[2] and Luísa Peixe[2]*

[1]*Faculdade de Ciências da Nutrição e Alimentação, Universidade do Porto, Rua Dr. Roberto Frias, 4200 Porto, Portugal;* [2]*REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, no. 164, 4050-047 Porto, Portugal*

*Corresponding author. Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, no. 164, 4050-047 Porto, Portugal. Tel: +351-22-2078972; Fax: +351-22-2003977; E-mail: lpeixe@ff.up.pt

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Objectives: To assess the presence of multidrug-resistant (MDR) *Salmonella* with human clinical relevance in pig farms from different regions of Portugal and to analyse their mobile genetic elements associated with antibiotic resistance.

Methods: Seventy-nine samples were collected from six piggeries and analysed for the presence of *Salmonella*. All isolates were examined for susceptibility to antimicrobial agents and representative isolates for resistance genes and class 1 integrons (PCR/restriction fragment length polymorphism). Clonality was determined by PFGE and multilocus sequence typing (MLST). Plasmid analysis included determination of size, content and characterization of the incompatibility group (rep-PCR and I-CeuI/S1-hybridization).

Results: Sixty *Salmonella* isolates were recovered from five samples (two manure, two waste lagoons and one animal feed) in half of the piggeries studied. All isolates were resistant to at least one antibiotic (tetracycline) and 97% to at least four antibiotics from different families. In 10 isolates representing different serogroup and resistance phenotype combinations a diversity of resistance genes and integrons was detected. These isolates belonged to the internationally widespread *Salmonella* Rissen (ST469) and *Salmonella* Typhimurium DT104 (ST19) clones, as well as to the emerging *Salmonella* Typhimurium monophasic variant with examples of Spanish (carrying a *sul3*-atypical integron within IncA/C plasmids, here assigned to ST19) and European (ASSuT phenotype, assigned to ST34) clones.

Conclusions: This is one of the few studies reporting emerging MDR *Salmonella* clones and the first one detecting *Salmonella* Typhimurium monophasic variant in the pig production setting. The survival of these strains in manure and waste lagoons is of concern, since these environments might allow spread of MDR bacteria beyond pig farms' boundaries.

Keywords: antimicrobial agents, piggeries, PFGE clones, MLST

Introduction

In recent years, changing trends in salmonellosis and associated serotypes have been observed, with a marked increase in certain multidrug-resistant (MDR) clones of *Salmonella* Typhimurium and its monophasic variant in different countries.[1,2] Among these, *Salmonella* Typhimurium DT104, OXA-30-producing *Salmonella* Typhimurium clone and *Salmonella* Typhimurium monophasic variant (4,[5],12:i:-) are the most frequently reported in Europe.[2,3] The latter, which have recently emerged worldwide, range from pan-susceptible (USA, Brazil) to MDR (Europe) strains and seem to be largely distributed in animal hosts and their derived products (e.g. pork, poultry products, cattle).[2] Although there is evidence that the animal setting seems to be a reservoir of MDR strains, data concerning the spread of emerging *Salmonella* MDR clones with features of clinical interest within and off pig farms are still missing. Here, we assessed the presence of MDR *Salmonella* with human clinical relevance on pig farms from different regions of Portugal and analysed their mobile genetic elements associated with antibiotic resistance.

Materials and methods

Sample processing and Salmonella identification

Seventy-nine samples were collected within six geographically separated Portuguese piggeries (five with intensive and one with extensive production) during 2006 and 2007. They included samples from pigs ($n=21$; faeces, nostril/surface swabs), feed/medicines ($n=22$; feed, water, medicine, antiseptics), residues ($n=17$; swine waste lagoons,

residual waters, manure, septic tank) and piggery facilities ($n=19$; water, walls/floors dust, soil). The presence of *Salmonella* was screened by the conventional method following ISO 6579, which includes two stages of enrichment and plating out in two selective solid media (four plates per sample).[4] Suspected colonies (up to five from each of the four plates) were identified by slide agglutination (*Salmonella* O poly antisera and serogroup-specific antisera for serogroups B, C1 and D; BD, USA), biochemical tests (API 32 GN; bioMérieux, Marcy l'Étoile, France) and a PCR assay (targeting the *invA* gene and a DT104/U302 phage type-specific DNA sequence).[5] The serotypes of representative isolates were determined at the National Centre of *Salmonella*. The *Salmonella* Typhimurium monophasic variant (4,[5],12:i:-) isolates were confirmed using PCR as previously described.[2]

Antimicrobial susceptibility testing

All *Salmonella* isolates were tested for susceptibility to 10 antimicrobial agents (μg) [amoxicillin (10), gentamicin (10), kanamycin (30), streptomycin (10), ciprofloxacin (5), nalidixic acid (30), chloramphenicol (30), tetracycline (30), sulfamethoxazole (300) and trimethoprim (5)] by the disc diffusion method following CLSI standards.[6] *Escherichia coli* ATCC 25922 was used as the control strain. Amoxicillin-resistant isolates were further tested for susceptibility to several extended-spectrum β-lactams (ceftazidime, ceftriaxone, cefotaxime, cefepime, cefoxitin, aztreonam and imipenem) and the double disc synergy test (DDST) for ESBL detection was also conducted.[6] From each sample, the selection of representative isolates for further studies was based on serogroup, presence of the *invA* gene and/or phage type DT104/U302 phage type-specific DNA sequence and antibiotic resistance phenotype.

Characterization of antimicrobial resistance genes, integrons and plasmids

Genes coding for resistance to sulfamethoxazole (*sul1*, *sul2* and *sul3*), tetracycline [*tet*(A), *tet*(B) and *tet*(G)], chloramphenicol (*floR*, *cmlA* and *catA*), amoxicillin (bla_{TEM}, bla_{PSE-1} and bla_{OXA-30}), gentamicin [*aac(3)-IV*], streptomycin (*aadA* and *strA-strB*) and trimethoprim (*dfrA1* and *dfrA12*) were searched for by PCR using primers and conditions previously described.[7,8] The detection and characterization of class 1 integrons was performed by PCR and restriction fragment length polymorphism (RFLP) analysis with TaqI as previously reported.[7,8] Positive and negative controls were included in all PCRs. Plasmid content and the genetic localization of integrons and *sul* genes were investigated by S1 nuclease (Takara Bio Inc., Shiga, Japan) and I-CeuI (New England BioLabs, Ipswich, MA, USA) digestion of total genomic DNA followed by PFGE. Identification of plasmid incompatibility groups was determined by a rep-PCR typing method[3] including three additional PCR assays for the IncU, IncR and ColE groups.[9] Southern blot hybridization was performed by standard methods using *intI1*, *sul1*, *sul2*, *sul3* and *rep* intragenic probes, following the manufacturer's instructions (Gene Images Alkphos Direct Labelling System Kit; Amersham GB/GE Healthcare Life Sciences UK Limited).

PFGE and MLST analysis

Clonal relatedness among isolates was assessed by PFGE following XbaI digestion of genomic DNA according to the standard 1 day protocol of the CDC.[7] *Salmonella enterica* serotype Braenderup H9812 (CDC) was used as a molecular size marker. Multilocus sequence typing (MLST) analysis was performed using specific primers to amplify a set of seven housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA* and *thrA*) and sequence type (ST) was assigned according to the MLST database (http://mlst.ucc.ie/mlst).

Results and discussion

Sixty *Salmonella* isolates were recovered from five positive samples (two manure, two waste lagoons and one animal feed) collected on three pig farms with intensive production (one located in the north and two located in the south of Portugal). Forty-seven isolates were serogroup B, of which 22 were positive for the DT104/U302 phage type-specific region, and 13 were serogroup C1. All isolates were resistant to at least one antibiotic (tetracycline) and 58 out of 60 were resistant to at least four antibiotics from different families (ranging from four to eight antibiotics). Resistances to tetracycline ($n=60$ isolates), streptomycin ($n=58$), sulfamethoxazole ($n=57$) and amoxicillin ($n=56$) were the most frequently detected and might reflect the high usage of these antibiotics in food-producing animals.[7] Lesser rates of resistance were observed for the other antimicrobial agents tested; chloramphenicol ($n=22$), trimethoprim ($n=19$), nalidixic acid ($n=10$), gentamicin ($n=8$), ciprofloxacin ($n=0$) and kanamycin ($n=0$). Susceptibility to extended-spectrum β-lactams and absence of extended-spectrum β-lactamases (ESBLs) and AmpC were observed. Because enrichment steps allowed selection of more than one isolate of the same strain from the same sample, 10 isolates representing different combinations of serogroup and antibiotic resistance phenotype were selected for further studies (Table 1). The selected isolates belonged to three *Salmonella* serotypes (four Rissen, two Typhimurium and four from its monophasic variant 4,[5],12:i:-) and four PFGE clones spread among different farms and samples (Figure 1 and Table 1). Among them a diversity of antibiotic resistance genes were detected. These strains harboured common MDR genetic elements (e.g. integrons) and shared clonal relationships with previously nationally widespread/emerging clones in human and food products,[7,8] showing their potential transmission along the food chain and their ability to cause human infections (Figure 1).

More than one resistant genotype or clone was detected within the same piggery, suggesting enrichment of the local metagenome with a broad range of drug-resistant strains and genetic elements, such as integrons. Isolates belonging to the emerging *Salmonella* Rissen clone ($n=4$), detected in the manure of two geographically separated piggeries, were assigned to ST469, only previously reported in the few European isolates of the same serotype allocated in the MLST database (http://mlst.ucc.ie/mlst). The human acquisition of *Salmonella* Rissen was previously associated with tourism or international trade outside the EU,[10] but the data of this and a few other studies suggest a more local source of infection with this emerging serotype.[7] Different resistance patterns and the presence of a specific class 1 integron (*dfrA12-orfF-aadA2*), located in IncR plasmids (35 kb), were observed, which may provide a selective advantage for this emergent serotype in the animal niche. Two isolates from the widely disseminated clone of *Salmonella* Typhimurium, DT104, showing the chromosomal located R-type ACSSuT (ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, tetracycline), were detected in environmental samples from one piggery (manure and waste lagoon), suggesting that, beside the classical dissemination routes (animals and foods of animal origin), environmental reservoirs might also play a role in the spread of such strains. Of particular interest was the

Table 1. Characterization of the *Salmonella* clones from Portuguese piggeries

Serotype (phage type)/ PFGE type[a]	No. of isolates	MLST – ST (no. of isolates)[b]	Date of isolation	Piggery	Sample	Resistance phenotype[c]/resistance genes profile (no. of isolates; sample)	Class 1 integron genes (bp)	Class 1 integron and/or *sul2* location Chr or PL (kb, Inc)[d]
Rissen/N	4	ST469 ($n=2$)	2006	B, C	manure B1; manure C7	AMX, STR, SUL, TET, TMP bla_{TEM}, *aadA2*, *sul1*, *tet*(A), *dfrA12* ($n=2$; B1 and C7)	*intI1*, *sul1* *dfrA12*, *orfF*, *aadA2* (2000)	PL (35, R)
						AMX, STR, TET, TMP bla_{TEM}, *tet*(A) ($n=1$; B1)	*intI1*	PL (30, R)
						TET *tet*(A) ($n=1$; C7)	—	—
Typhimurium (DT104/ U302)/A	2	ST19 ($n=1$)	2007	E	waste lagoon E32; manure E34	AMX, CHL, STR, SUL, TET bla_{PSE-1}, *floR*, *aadA2*, *sul1*, *tet*(G) ($n=2$; E32 and E34)	*intI1*, *sul1* *aadA2* (1000) *blaPSE-1* (1200)	Chr
Typhimurium (DT104/ U302) 4,[5],12:i:-/O	2	ST19 ($n=1$)	2006	C	manure C7	AMX, CHL, GEN, NAL, STR, SUL, TET, TMP bla_{TEM}, *cmlA1*, *aac(3)-IV*, *aadA2*, *sul1-sul2-sul3*, *tet*(A), *dfrA12* ($n=1$; C7)	*intI1*, *sul1* *dfrA12*, *orfF*, *aadA2* (2000) type III-*sul3*[e]	PL (170, A/C)
						CHL, NAL, STR, TET, SUL *cmlA1*, *aadA*, *tet*(A), *sul1-sul2-sul3* ($n=1$; C7)	*intI1*, *sul1* type III-*sul3*[e]	PL (130, A/C)
Typhimurium 4,[5],12:i:-/Y	2	ST34 ($n=2$)	2007	E	feed E2; manure E34	AMX, STR, SUL, TET bla_{TEM}, *strA-strB*, *sul2*, *tet*(B) ($n=2$; E2 and E34)	—	Chr (*sul2*)

[a]Clones are designated by capital letters, as previously published.[7,8]
[b]Number of isolates submitted to MLST database.
[c]AMX, amoxicillin; CHL, chloramphenicol; GEN, gentamicin; NAL, nalidixic acid; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TMP, trimethoprim.
[d]Chromosomal (Chr) and/or plasmid (PL) location of integrons and the *sul2* gene was assessed by hybridization of I-CeuI/S1-digested genomic DNA using *int1*, *sul1*, *sul2*, *sul3* and *rep* probes.
[e]Structure of the type III *sul3* integron: 5′*CS-estX-psp-aadA2-cmlA1-aadA1-qacH-IS440-sul3*.[8]

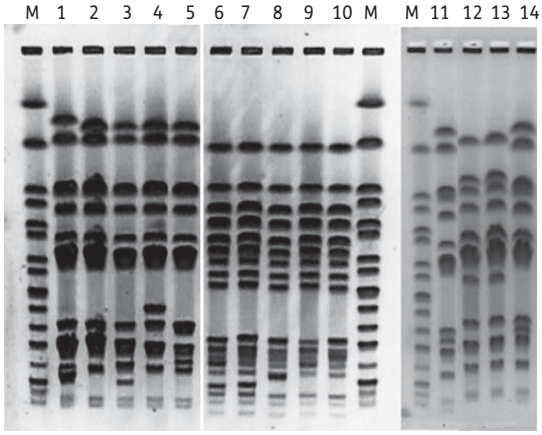

Figure 1. PFGE patterns of *Salmonella* isolates from piggeries and others previously characterized.[7,8] Lanes: M, *Salmonella* Braenderup H9812 (CDC); 1, clone O, human isolate (*Salmonella* Typhimurium 4,[5],12:i:-, 459/02); 2, clone O, pork isolate (*Salmonella* Typhimurium 4,[5],12:i:-, A30); 3, C7.5 isolate (*Salmonella* Typhimurium 4,[5],12:i:-, piggery C; clone O); 4, C7.17 isolate (*Salmonella* Typhimurium 4,[5],12:i:-, piggery C; clone O); 5, clone O, pork isolate (*Salmonella* Typhimurium 4,[5],12:i:-, B296); 6, C7.7 isolate (*Salmonella* Rissen, piggery C; clone N); 7, B1.1 isolate (*Salmonella* Rissen, piggery B; clone N); 8, clone N, pork isolate (*Salmonella* Rissen, A27); 9, C7.16 isolate (*Salmonella* Rissen, piggery C; clone N); 10, B1.3 isolate (*Salmonella* Rissen, piggery B; clone N); 11, E32.2 isolate (*Salmonella* Typhimurium DT104, piggery E; clone A); 12, E2.5 isolate (*Salmonella* Typhimurium 4,[5],12:i:-, piggery E; clone Y); 13, E34.1 isolate (*Salmonella* Typhimurium 4,[5],12:i:-, piggery E; clone Y); and 14, E34.11 isolate (*Salmonella* Typhimurium DT104, piggery E; clone A).

detection of two MDR clones of the emerging *Salmonella* Typhimurium monophasic variant, related to the so-called European and Spanish clones.[11,12] The pattern ASSuT was observed in the isolates from one piggery associated with the chromosomal resistance genes [bla_{TEM-1}, *strA-strB*, *sul2* and *tet*(B)] typical of the European monophasic strains,[12] here demonstrated to belong to ST34 (a single-locus variant of ST19). The other monophasic variant MDR clone, belonging to the worldwide-distributed ST19 and DT104/U302 phage type, presented an IncA/C plasmid (130–170 kb) with the recently characterized atypical *sul3*-carrying integron type III.[8] Interestingly, isolates of this monophasic variant clone carrying similar MDR features have been recovered for more than 10 years in Spain[11] and from humans and food products in Portugal, and were initially identified as *Salmonella* Typhimurium.[8] Successful adaptation of this pathogen and the intense commercial trade in live pigs and pig meat products between these countries might explain the maintenance and dissemination of the monophasic MDR so-called Spanish clone in the Iberian Peninsula.

This study indicates that clinically relevant MDR clones are prevalent and disseminated in piggeries. The success of these bacterial strains might be related to antimicrobial resistance features, but also to other properties, such as virulence factors, as recently described for the IncA/C plasmids in the Spanish clone.[11] Also, the detection of these widely frequent MDR genetic lineages of *Salmonella* in manure and swine waste lagoons raises concerns due to potential water, air and soil contamination, as well as of wild animals, indicating potentially high risks of transmission of the pathogen in the environment, animals and humans.[13–15] The frequent use of manure as a fertilizer and the long-term survival potential of this zoonotic pathogen seem to contribute to the increasing reports of salmonellosis outbreaks caused by consumption of fresh products (e.g. ready-to-eat salads).[13,14] This alerts us to the ability of the emergent clones, such as subtypes of *Salmonella* Typhimurium monophasic variant to be transmitted to humans by sources other than pigs and pork, until now considered sporadic.

In conclusion, this is one of the few studies reporting emerging MDR *Salmonella* clones carrying features with clinical interest and the first one identifying *Salmonella* Typhimurium monophasic variant in pig farms. The presence of isolates resistant to several antibiotic families and belonging to clonal lineages increasingly associated with human infections, particularly the emergence of *Salmonella* Typhimurium monophasic variants in piggeries, is a public health threat.

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Transparency declarations

None to declare.

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Characterization of the emerging clinically-relevant multidrug-resistant *Salmonella enterica* serotype 4,[5],12:i:- (monophasic variant of *S.* Typhimurium) clones

Joana Mourão[1], Jorge Machado[2], Carla Novais[1], Patrícia Antunes[1,3] and Luísa Peixe[1]*

[1] REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal; [2] Laboratório Nacional de Referência de Infeções Gastrintestinais, Departamento de Doenças Infeciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisboa, Portugal; [3] Faculdade de Ciências da Nutrição e Alimentação, Universidade do Porto, Porto, Portugal.

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ARTICLE

Characterization of the emerging clinically-relevant multidrug-resistant *Salmonella enterica* serotype 4,[5],12:i:- (monophasic variant of *S.* Typhimurium) clones

J. Mourão · J. Machado · C. Novais · P. Antunes · L. Peixe

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Abstract To better understand the recent success/emergence of *Salmonella enterica* serotype 4,[5],12:i:- we characterized the population diversity, *flj*AB deletion patterns, antibiotic resistance features and associated genetic elements of a comprehensive collection obtained in the last decade from Portugal (2002–2010). One hundred thirty-one isolates from human clinical specimens, food, environment and piggeries, verified by PCR as *S.* 4,[5],12:i:-, were studied for clonality (Pulsed-Field Gel Electrophoresis and Multilocus Sequence Typing), antibiotic resistance by phenotypic (disk diffusion and/or agar dilution) and genotypic (PCR/Restriction Fragment Length Polymorphism and sequencing, genomic location) methods and *fljAB*-deletions (PCR). Plasmid analysis included determination of size, content and characterization of the incompatibility group (PCR-Based Replicon Typing and I-*Ceu*I/S1-hybridization). Results showed three multidrug-resistant (MDR) clones circulating and causing infections, associated with particular phenotypic and genotypic features. Most of the isolates belonged to the widespread European (ASSuT phenotype, RR1-RR2 resistance regions, ST34) and Spanish (carrying a *sul3*-type III integron within IncA/C plasmids, ST19) clones circulating in Europe. A third clone, here designated Southern European clone (carrying a *sul3*-type I integron within IncR plasmids, ST19), presents a *fljAB* region different from the previous clones and similar to the US strains, despite differences in the MDR mobile genetic platforms. The success of *S.* 4,[5],12:i:- might be related to the selective advantage offered by MDR profiles associated with stable genetic elements, also carrying virulence features, along with well adapted clones to the animal food production and causing human infections.

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J. Mourão · C. Novais · P. Antunes · L. Peixe
REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal

J. Machado
Laboratório Nacional de Referência de Infeções Gastrintestinais, Departamento de Doenças Infeciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisboa, Portugal

P. Antunes (✉)
Faculdade de Ciências da Nutrição e Alimentação, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal
e-mail: patriciaantunes@fcna.up.pt

Introduction

Non-typhoidal *Salmonella* is one of the leading causes of zoonotic food borne diseases, presenting public health relevance, including such industrialized regions as the European Union (EU) [1, 2]. Currently, changing trends in salmonellosis and associated serotypes have been observed, with predominant multidrug resistant (MDR) *Salmonella* clonal lineages scattered worldwide in a wide range of food animals and involved in endemic/epidemic scenarios, such as described for *Salmonella enterica* serotype 4,[5],12:i:- [1, 3]. This serotype is a monophasic variant of the recognized pathogenic *Salmonella enterica* serotype Typhimurium and has become a new epidemic strain causing human infections transmitted by the food chain, suggesting good adaptability to different environmental conditions and colonization/infection of a wide range of animals and humans [1, 3–5].

In Europe, including Portugal, *S.* 4,[5],12:i:- has been the third most frequent serotype among human salmonellosis [1] and responsible for several recent large outbreaks [6, 7]. Food animals, particularly pigs, were pointed out as its reservoir and their derived products as the main infection vehicles [3, 8, 9]. Those *S.* 4,[5],12:i:- isolates present MDR profiles associated with particular widespread clonal lineages, designated

European and Spanish clones [3, 4]. However, few studies comprehensively analyse the genetic elements (genes, integrons, plasmids) spreading antibiotic resistance [10–12] and the *flj*AB genomic deletion patterns in *S.* 4,[5],12:i:- [13]. This serotype is currently targeted by EU actions to detect and control *Salmonella* serotypes of concern [14], requiring further surveillance and mitigation strategies, including its epidemiological success characterization. To better understand the recent emergence of *S.* 4,[5],12:i:- we characterized the population diversity, antibiotic resistance features and associated genetic elements, plus *flj*AB deletion patterns, of a comprehensive collection obtained in the last decade from Portugal.

Materials and methods

Bacterial isolates

A total of 131 isolates of *S.* 4,[5],12:i:- were studied, with isolates recovered from human clinical specimens (n=114; faeces, blood, peritoneal fluid and urine; from 28 hospitals and two community laboratories), food of animal origin (n=9; pork, beef and poultry), environment (n=4; drinking and bathing water) and piggeries (n=4; manure and feed) between 2002 and 2010 from different geographical areas of Portugal. Those isolates were serologically identified as *S.* 4,[5],12:i:- by the National Reference Centre (INSA, Lisbon, Portugal) and confirmed by PCR targeting the intergenic region *fliB-fliA* and *fljB* gene coding for phase-2 flagellar antigen [3, 15]. For evolutionary analyses, all isolates were screened for genomic deletions patterns surrounding *fljAB* operon (*fljA*, *fljB*, *hin, iroB,* STM2740 and STM2757 genes and STM1053-1997 region) by PCR [13]. Also, isolates were screened by multiplex PCR targeting *invA* gene and DT104/U302 phage-type-specific DNA [16].

Molecular subtyping

Clonal relatedness among isolates was established by pulsed-field gel electrophoresis (PFGE) of *Xba*I-digested genomic DNA [8]. Band patterns were analysed with InfoQuest™ FP software v4.5 (BioRad Laboratories) and cluster analysis with unweighted pair group method with arithmetic averages based on Dice similarity coefficient with 1.0 % optimization and tolerance. Isolates with Dice band-based similarity coefficient ≥85 % were considered to belong to the same PFGE-type (designated by capital letters). Multilocus sequence typing (MLST) was also performed in representative isolates (n=16; different PFGE-types/clones) (http://mlst.ucc.ie/mlst/dbs/Senterica).

Study of antibiotic susceptibility and resistance genes

Susceptibility to amoxicillin, gentamicin, kanamycin, streptomycin, ciprofloxacin, nalidixic acid, chloramphenicol, tetracycline, sulfamethoxazole and trimethoprim was determined by disc diffusion or agar dilution methods (ciprofloxacin) following CLSI guidelines [17]. Amoxicillin resistant isolates were further tested for several extended spectrum β-lactams (ceftazidime, cefotaxime, cefepime and cefoxitin) and by double disk synergy test for ESBL [17]. MDR was considered when isolates were resistant to three or more antibiotics of different families. The search for genes encoding resistance to amoxicillin (bla_{TEM}), chloramphenicol (*catA, cmlA1* and *floR*), ciprofloxacin [*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA*, *oqxA*, *oqxB* and *aac(6)'-Ib-cr*], gentamicin [*aac(3)-IV*], kanamycin (*aphA*) streptomycin (*strA-strB*, *aadA*), sulfamethoxazole (*sul1*, *sul2*, *sul3*), tetracycline [*tet*(A), *tet*(B), *tet*(G)] and trimethoprim (*dfrA*1, *dfrA*12) was performed by PCR [8]. Detection and characterization of class 1 integrons was performed by PCR and restriction fragment length polymorphism (*Taq*I) [18, 19]. Representative isolates (n=22; different PFGE-types) were also PCR tested for presence of left, right and internal regions of genomic resistance region RR1 (RR1LJ, RR1RJ, *tnpB* and *repC* regions/genes) and RR2 (RR2LJ, RR2RJ, IS*10* and *tetC*(B)Δ regions/genes) [11]. A positive amplicon from each PCR was sequenced and compared with known GenBank sequences to validate the method.

Characterization of the genetic elements carrying antibiotic resistance genes

Conjugation assays were done for isolates representative of different PFGE-types using *Escherichia coli* K802N (rifampicin and nalidixic acid resistant) as recipient strain [19]. Transconjugants were selected on Mueller-Hinton 2 agar containing nalidixic acid (64 mg/L) plus tetracycline (8 mg/L) or sulfamethoxazole (256 mg/L). Classification of plasmids was based on PCR-based replicon typing [20, 21] and location of antibiotic resistance genes by hybridization of S1-PFGE and I-*Ceu*I-PFGE with specific probes [8, 19].

Results

Typing of *Salmonella* 4,[5],12:i:- isolates

The 131 isolates corresponded to 35 PFGE profiles, which were grouped into ten PFGE-types and three clones (showing 67.8 to 75.2 % identity) (Online Resource 1). Two showed phenotypic and genotypic features similar to previously described European and Spanish clones (see further sections). A third one presented unique features and was here designated

Southern European clone. Within the European clone were included 50 % of monophasic strains, non DT104/U302 phage-type. They were classified in three prevalent PFGE-types, with type-A gathering strains from humans, environment and piggeries (Table 1). The Spanish clone (DT104/U302 phage-type) included 35 % of the isolates, dominated by PFGE type O (n=44), including also human, environment, food and piggeries isolates. Finally, the less prevalent Southern European clone includes 15 % of the isolates of human and food origins, all non DT104/U302 phage-type (Table 1). The MLST typing assigned Spanish and Southern European clones in ST19 and the European clone in its single-locus variant ST34. Also, genomic deletions patterns surrounding the *fljAB* region were constant in all isolates from MDR Spanish and Southern European clones, but diverse in European clone isolates, in spite of 70 % (n=46/66) presenting a predominant profile (Table 1).

Antibiotic resistance study

All but two *S.* 4,[5],12:i:- isolates (n=129/131; 98 %) were resistant to at least one of the antibiotics tested. They expressed resistance to sulfamethoxazole (n=121/92 %), tetracycline (n=119/91 %), streptomycin (n=119/91 %), amoxicillin (n=89/68 %), chloramphenicol (n=60/46 %), trimethoprim (n=46/35 %), gentamicin (n=35/27 %) and kanamycin (n=4/3 %). Decreased susceptibility to ciprofloxacin (n=6/5 %), with (n=4) or without (n=2) resistance to nalidixic acid, was only observed in isolates from PFGE-types B (European clone) and O (Spanish clone), but none carried the plasmid-mediated quinolone resistance genes screened. Susceptibility to extended-spectrum β-lactams and absence of ESBL/AmpC were also observed.

Diverse antibiotic resistance profiles (ranging from one to eight antibiotics) were identified among isolates belonging to the three clones, with 92 % (n=121/131) resistant to three or more antibiotics. The most frequent profiles were ASSuT (amoxicillin, streptomycin, sulfamethoxazole, tetracycline) typical of the European clone (n=51/66), ACGSSuTTm (amoxicillin, chloramphenicol, gentamicin, streptomycin, sulfamethoxazole, tetracycline, trimethoprim) typical of the Spanish clone (n=26/46) and CSSuTTm (chloramphenicol, streptomycin, sulfamethoxazole, tetracycline, trimethoprim) on the Southern European clone (n=15/19) (Table 1).

Genetic elements carrying antibiotic resistance genes

Isolates of the European clone presented the typical antibiotic resistance genes [$bla_{\text{TEM-1}}$, *strA-strB*, *sul2* and *tet*(B)] chromosomally located and non-transferable. Absence of class 1 integrons in all but two isolates was also detected (Table 1). None of the tested isolates from this clone (n=22; all ASSuT) was simultaneously positive for presence of left, right and internal genes/regions of chromosomal genomic regions RR1 (RR1LJ$^+$ - 32 %, RR1RJ$^+$ - 18 % regions and *tnpB*$^+$ - 55 %, *repC*$^+$ - 73 % genes) and RR2 (RR2LJ$^+$ - 14 %, RR2RJ$^+$ - 95 % regions and IS*10*$^+$ - 73 %, *tetC*(B)Δ - 77 % genes). Although it was not possible to establish an association between those profiles and specific PFGE-types, six out of nine isolates from PFGE-type A exhibited particular patterns (RR1LJ + *tnpB* + *repC* + *tetC*(B)Δ + RR2RJ, n=4 and *tnpB* + *repC* + RR1RJ + RR2LJ + IS*10* + RR2RJ, n=2).

In contrast, the Spanish and Southern European clones, despite being included in the same ST19 lineage, carried different atypical class 1 integrons [*sul3*-type III (5′CS-*estX*-*psp*-*aadA*2-*cmlA*1-*aadA*1-*qacH*-IS440-*sul3*) on Spanish clone; *sul3*-type I (5′CS-*dfrA*12-*orfF*-*aadA*2-*cmlA*1-*aadA*1-*qacH*-IS440-*sul3*) on Southern European clone], located in the non-transferable plasmids, IncA/C (110–220 Kb) or IncR (80–160 Kb), respectively. Furthermore, most isolates belonging to the Spanish clone also carried one typical class 1 integron (*dfrA12-orfF-aadA2-qacE*Δ1-*sul1*), *tet*(A), *aac*(3)-IV and bla_{TEM} within the same plasmid (Table 1). None of the Southern European clone isolates exhibited resistance to amoxicillin (bla_{TEM}) and the tetracycline resistance was associated with *tet*(B) located in IncR plasmids.

Discussion

A decreasing trend in human salmonellosis cases is observed in European countries, mainly due to successful control programs in poultry/eggs production [1]. Nevertheless, occurrence of MDR clonal lineages causing human infections and resident in food animal settings becomes increasingly frequent, with the emergent *S.* 4,[5],12:i:- suggested as a new epidemic serotype in Europe [3, 4, 8]. In Portugal, the number of reported cases associated with this monophasic variant of *S.* Typhimurium had a nine-fold increase during this study period and nowadays ranks third in frequency (1,1 % in 2002 versus 22.6 % in 2010) [22], similar to other European countries [1, 3].

This study reports high rates (92 %) of MDR in *S.* 4,[5],12:i:- isolates from different origins and geographical regions of Portugal. Resistance to sulfamethoxazole, tetracycline, streptomycin and amoxicillin were the most frequent and might reflect their high use in food-production animals in Europe [23, 24]. Most of *S.* 4,[5],12:i:- isolates studied were grouped in the European clone/ST34, corroborating its recent epidemic emergence in Europe [4, 9, 25, 26] and, more recently, also in America [5, 27], which in all cases presented similar phenotypic and genotypic ASSuT markers. Nevertheless, a considerable diversity in PFGE-types is currently disseminated in Europe, particularly STYMXB.0131, STYMXB.0079 and STYMXB.0010 (PulseNet Europe) [4, 9,

Table 1 Genotypic and phenotypic characterization of *Salmonella enterica* serotype 4,[5],12:i:- clones from Portugal

Clone designation Phage type[a] MLST-ST (eBG) (no. isolates)[b] Predominant *fljAB* deletion pattern[c]	PFGE-type (no. isolates/ no. PFGE subtypes)[d]	Source (no. isolates)	Year/ region[e]	Resistance phenotype (no. isolates)[f] / Resistance genes profile	Class 1 integron (bp)[i] (no. isolates)	Class 1 integron and/or *sul2* location Chr or PL (Kb, Inc group)[j] (no. isolates)
European Clone	A (*n*=29; 5)	Human (*n*=26; 16 hospitals; 2 community); Environment (*n*=1; drinking water); Piggeries (*n*=2; manure, animal feed)	2003-2010 /N, C, S	A, S, Su, T, Tm (*n*=1)/*bla*$_{TEM}$, *aadA2*, *sul1*-*sul2*, *tet*(B), *dfrA1*	*dfrA1*-*orfF*-*aadA2* (1500) (*n*=1)	PL (55, I1) (*n*=1) and Chr (*sul2*) (*n*=4)
non DT104/U302				A, S, Su, T (*n*=19)/*bla*$_{TEM}$, *strA*-*strB*, *sul2*, *tet*(B)	(–) (*n*=28)	
ST34 (1) (*n*=6)				A, S, Su (*n*=4)/*bla*$_{TEM}$, *strA*-*strB*, *sul2*		
STM2740^{+}, STM2757$^{+/-}$, STM1053-1997^{-}, *fljA*$^{-}$, *fljB*$^{-}$, *hin*$^{-}$, *iroB*$^{+}$				S, Su, T (*n*=1)/*strA*-*strB*, *sul2*, *tet*(B)		
				A, T (*n*=2)/*bla*$_{TEM}$, *tet*(B) T (*n*=1)/*tet*(B) (–) (*n*=1)[g]		
	B (*n*=17; 5)	Human (*n*=17; 10 hospitals)	2008-2010/N, C, S, I	A, S, Su, T (C, G, Tm) (*n*=16)/ *bla*$_{TEM}$, *strA*-*strB*, *sul2*, *tet*(B) (*floR*, *dfrA12*, *aadA2*, *sul1*)[h] T (*n*=1)/*tet*(B)	*dfrA12*-*orfF*-*aadA2*[h] (2000) (*n*=1) (–) (*n*=16)	PL (230, HI2)[h] (*n*=1) and Chr (*sul2*) (*n*=8)
	C (*n*=19; 5)	Human (*n*=19; 15 hospitals)	2007-2010/N, C, S, I	A, S, Su, T (K) (*n*=14)/*bla*$_{TEM}$, *strA*-*strB*, *sul2*, *tet*(B)	(–) (*n*=19)	Chr (*sul2*) (*n*=5)
				A, S, Su (*n*=1)/*bla*$_{TEM}$, *strA*-*strB*, *sul2*		
				S, Su, T (*n*=3)/*strA*-*strB*, *sul2*, *tet*(B) T (*n*=1)/*tet*(B)		
	D (*n*=1; 1)	Human (*n*=1; 1 hospital)	2009/N	A, S, Su, T (*n*=1)/*bla*$_{TEM}$, *strA*-*strB*, *sul2*, *tet*(B)	(–) (*n*=1)	Chr (*sul2*) (*n*=1)
Spanish clone	O (*n*=44; 8)	Human (*n*=30; 20 hospitals); Food (*n*=8; poultry, pork, beef); Environment (*n*=3; bathing water); Piggery (*n*=2; manure); Unknown (*n*=1)	2002-2010/N, C, S	A, C, G, S, Su, T, Tm (K, Nx) (*n*=24)/*bla*$_{TEM}$, *cmlA1*, *aac*(3)-IV, *aadA2*-*aadA1*, *sul1*-*sul2*-*sul3*, *tet*(A), *dfrA12* (*floR*)	*dfrA12*-orfF-*aadA2* (2000) (*n*=27)	PL (110–220, A/C) (*n*=8)

Table 1 (continued)

Clone designation Phage type[a] MLST-ST (eBG) (no. isolates)[b] Predominant *fljAB* deletion pattern[c]	PFGE-type (no. isolates/ no. PFGE subtypes)[d]	Source (no. isolates)	Year/ region[e]	Resistance phenotype (no. isolates)[f] / Resistance genes profile	Class 1 integron (bp)[i] (no. isolates)	Class 1 integron and/or *sul2* location Chr or PL (Kb, Inc group)[j] (no. isolates)
DT104/U302				A, C, G, S, Su, Tm (*n*=2)/bla_{TEM}, *cmlA*1, *aac*(3)-IV, *aadA*2-*aadA*1, *sul*1-*sul*2-*sul*3, *dfrA*12 (*floR*)	Type III-*sul3* (7304) (*n*=43) (−) (*n*=1)	PL (170; A/C, N) (*n*=1)
ST19 (1) (*n*=5)				A, C, G, S, Su, T (*n*=2)/bla_{TEM}, *cmlA*1, *aac*(3)-IV, *aadA*2-*aadA*1, *sul*1-*sul*2-*sul*3, *tet*(A)		
STM2740^{+}, STM2757^{+}, STM1053-1997^{-}, *fljA*$^{-}$, *fljB*$^{-}$, *hin*$^{-}$, *iroB*$^{-}$				A, C, S, Su, T, Tm (*n*=1)/bla_{TEM}, *cmlA*1, *aadA*2-*aadA*1, *sul*1-*sul*2-*sul*3, *tet*(A), *dfrA*12 C, G, Su, T (S) (*n*=4)/*cmlA*1, *aac*(3)-IV, *sul*1-*sul*2-*sul*3, *tet*(A), *aadA*2-*aadA*1 C, S, Su, T (Nx) (*n*=7)/*cmlA*1, *aadA*2-*aadA*1, *sul*1-*sul*2-*sul*3, *tet*(A) C, G, S, Su (*n*=1)/*cmlA*1, *aac*(3)-IV, *aadA*2-*aadA*1, *sul*1-*sul*2-*sul*3 C, S, Su (*n*=1)/*cmlA*1, *aadA*2-*aadA*1, *sul*1-*sul*2-*sul*3 S, Su, T (*n*=1)/*aadA*2-*aadA*1, *sul*1-*sul*2-*sul*3, *tet*(A) Su (*n*=1)/*sul*1-*sul*2		
	X (*n*=2; 1)	Human (*n*=2; 1 hospital)	2004/S	A, C, G, S, Su, T, Tm (*n*=2)/bla_{TEM}, *cmlA1*, *aac*(3)-IV, *aadA*2-*aadA*1, *sul*1-*sul*2-*sul*3, *tet*(A), *dfrA*12	*dfrA*12-*orfF*-*aadA*2 (2000) (*n*=2) Type III-*sul3* (7304) (*n*=2)	PL (130, A/C) (*n*=1)
Southern European Clone	Q (*n*=9; 4)	Human (*n*=8; 8 hospitals); Food (*n*=1; pork)	2004, 2006–2010/N, C, S	C, S, Su, T, Tm (*n*=7)/*cmlA*1, *aadA*2-*aadA*1, *sul*3, *tet*(B), *dfrA*12 (*strA*-*strB*) T (*n*=2)/*tet*(B)	Type I-*sul3* (7085) (*n*=7) (−) (*n*=2)	PL (80–160, R) (*n*=3)

Table 1 (continued)

Clone designation Phage type[a] MLST-ST (eBG) (no. isolates)[b] Predominant *fljAB* deletion pattern[c]	PFGE-type (no. isolates/ no. PFGE subtypes)[d]	Source (no. isolates)	Year/ region[e]	Resistance phenotype (no. isolates)[f] / Resistance genes profile	Class 1 integron (bp)[i] (no. isolates)	Class 1 integron and/or *sul2* location Chr or PL (Kb, Inc group)[j] (no. isolates)
non DT104/U302	G (*n*=7; 3)	Human (*n*=7; 6 hospitals)	2003-2004, 2009–2010/N	C, S, Su, T, Tm (*n*=7)/*cmlA*1, *aadA*2-*aadA*1, *sul*3, *tet*(B), *dfrA*12 (*strA-strB*)	Type I-*sul3* (7085) (*n*=7)	PL (110–140, R) (*n*=5)
ST19 (1) (*n*=5)	F (*n*=2; 2)	Human (*n*=2; 2 hospitals)	2008-2009/N	C, S, Su, T, Tm (*n*=1)/*cmlA*1, *aadA*2-*aadA*1, *sul*3, *tet*(B), *dfrA*12 (*strA-strB*) T (*n*=1)/*tet*(B)	Type I-*sul3* (7085) (*n*=1) (−) (*n*=1)	PL (nd, R) (*n*=1)
STM2740^{-}, STM2757^{-}, STM1053-1997^{+}, *fljA*$^{-}$, *fljB*$^{-}$, *hin*$^{+}$, *iroB*$^{+}$	E (*n*=1; 1)	Human (*n*=1; 1 hospital)	2007/S	(−) (*n*=1)[g]	(−) (*n*=1)	(−)

[a] PCR assay for the identification of *Salmonella enterica* serotype Typhimurium DT104/U302 [16]

[b] ST, sequence type; eBG, eBurst Groups identified by Multilocus Sequence Typing. Number of isolates submitted to *Salmonella* MLST Database

[c] Pattern of genomic deletions surrounding the *fljAB* operon, including *fljA*, *fljB*, *hin, iroB,* STM2740 and STM2757 genes and STM1053-1997 region by PCR according to Soyer et al. [13]; +, presence; −, absence

[d] Clones are designated by capital letters

[e] N, North; C, Centre; S, South and I, Islands (Azores)

[f] Variable presence of resistance phenotype and genotype is indicated in parenthesis. Antimicrobial abbreviations: A, amoxicillin; C, chloramphenicol; Cp, ciprofloxacin; G, gentamicin; K, kanamycin; Nx, nalidixic acid; S, streptomycin; Su, sulfamethoxazole; T, tetracycline; Tm, trimethoprim

[g] (−), susceptible to all the antibiotics tested

[h] The transferability of underlined genes was only verified in one isolate

[i] (−), absence of class 1 integron. Atypical integron characterized: Type I-*sul*3 (5′CS-*dfrA*12-*orfF*-*aadA*2-*cmlA*1-*aadA*1-*qacH*-IS440-*sul*3); Type III-*sul3* (5′CS-*estX*-*psp*-*aadA*2-*cmlA*1-*aadA*1-*qacH*-IS440-*sul*3) [19]

[j] Chromosomal (Chr) and/or plasmid (PL) location of integron and/or the *sul*2 genes was assessed by hybridization of I-*Ceu*I/S1-digested genomic DNA using *int*I1, *sul*1, *sul*2, *sul*3 and rep probes; nd, not determined

25, 26]. Here, the dominant PFGE-type A, corresponding to STYMXB.0079 profile particularly widespread in Italy [11, 25], was found in human clinical samples and piggeries (manure and animal feed), suggesting that pig-production may be its main reservoir in Portugal, as demonstrated for other European *S.* 4,[5],12:i:- PFGE-types (e.g., STYMXB.0131 in Germany; STYMXB.0010 in Greece) [9, 26]. Common pig breeding lines and commercial trade in living animals, feed and meat products could also explain the spread of these MDR clones in Europe [28]. The ASSuT pattern was detected in all PFGE-types and was associated with chromosomal resistance genes [$bla_{\text{TEM-1}}$, *strA-strB*, *sul2* and *tet*(B)], recently localized in two highly conserved resistance regions in STYMXB.0079-ASSuT strains [11]. However, the detection of isolates with other resistant patterns (ASSu, SSuT, AT, T) along with diverse results obtained for RR1 and RR2 left, right and internal regions in ASSuT isolates, suggests rearrangements or deletions within those chromosomal resistance regions of the European clone. Furthermore, we found several patterns of genomic deletions surrounding the *fljAB* region, including those previously described for European clone isolates [11] or in atypical US strains (32 % of our isolates) [13], suggesting that multiple independent emergence events occurred in ST34 clonal lineage, most likely from *S.* Typhimurium.

The isolates belonging to the Spanish clone presented phenotypic and genotypic markers previously described [10, 29]. This clonal lineage, originally identified in Spain where it still predominates [10, 29], was subsequently reported in food products, environment or piggeries and is causing human infections in neighboring Portugal since 2002 [8, 19]. Intense commercial trade between Portugal and Spain might explain the maintenance and dissemination of the Spanish clone in the Iberian Peninsula. Most isolates studied presented the core ACGSSuTTm R-type, related with class 1 integrons, located on large non-transferable IncA/C or IncA/C-N plasmids, as described in Spain [10]. Acquisition of those epidemic plasmids, carrying multiple antibiotic resistance determinants and/or virulence genes [10, 30], could contribute to the Spanish clone maintenance and spread. Moreover, all MDR isolates carried the same genomic deletion pattern surrounding the *fljAB* region as described for few Spanish isolates studied, suggesting that this clone represents a distinct genetic lineage that evolved through an independent event from *S.* Typhimurium [13].

Of note, is the description of a third MDR *S.* 4,[5],12:i:- group within the worldwide distributed ST19, circulating, at least, since 2003 in Portugal and initially identified as *S.* Typhimurium [19]. Their discrimination from European and Spanish clones comprises several features, including PFGE profiles, MDR phenotypes and plasmid-integron encoded resistance determinants. Moreover, when tested by PCR according to Soyer et al. [13] the same particular genomic deletion pattern surrounding the *fljAB* operon was found in all isolates, presenting *hin* and *iroB* genes and the STM1053-1997 region (all absent from Spanish clone) but not *fljA*, *fljB*, STM2740 and STM2757 genes. This pattern is also shared by ST19 USA strains [13], although IncR plasmids and MDR profile characterizing our isolates were absent (reference strain CVM23701, GenBank accession no. NZ_ABAO00000000.1). These data suggest a common evolutionary origin for US strains and Southern European clone, with the acquisition of MDR IncR plasmids by the latter, possibly driven by antibiotic selective pressure and availability of IncR in the European metagenome [8, 21, 31]. Moreover, all but three Southern European clone isolates presented the MDR pattern CSSuTTm associated with a genetic platform including an atypical *sul3* class 1 integron, previously described in Portuguese isolates [19], and *tet*(B), both located on large non-transferable IncR plasmids. Identification of those features in few sporadic *S.* 4,[5],12:i:- Italian (human) and Spanish (swine) isolates [12] suggests that this clone might be spread in other countries. IncR plasmids have been described in different *Enterobacteriaceae* and associated with high-risk clones and/or clinically relevant genes (e.g. bla_{VIM}, $bla_{\text{CTX-M}}$, and *qnr* genes) [21, 30, 31], including in the animal setting (e.g. *S.* Rissen in Portuguese piggeries) [8]. Simultaneous presence of resistance genes to different antibiotics and/or virulence [12] could contribute to successful persistence of IncR plasmids by diverse co-selection events and provide selective advantages for this emerging *S.* 4,[5],12:i:- clone. Its potential reservoir and transmission pathways (e.g., food and animal sources, travel outside European Union), however, remain unknown and require further studies.

In summary, in this nine-year period study, we identified three *S.* 4,[5],12:i:- clones of particular PFGE-types, specific MDR profiles and genetic elements circulating in Portugal, with two of those related to the highly disseminated European and Spanish clones. We described a third successful MDR clonal lineage associated with human infections, originated by a *fljAB*-deletion event distinct from the European and Spanish clones and similar to the US strains, carrying several clinically important antibiotic resistance genes located in newly described IncR plasmids, which could contribute to higher prevalence of *Salmonella* serotype 4,[5],12:i:-. Moreover, a combinaton of molecular features typical of each clone (e.g., PFGE-type, *sul3*-type I integron and IncR-Southern European clone; PFGE-type, *sul3*-type III integron and IncA/C-Spanish clone; PFGE-type, ASSuT-RR1/RR2-European clone) may constitute biomarkers for tracing MDR *Salmonella* 4,[5],12:i:- clones with potential for clinical diagnosis and epidemiological surveillance. Nevertheless, further studies are needed to assess the contribution of other driving forces (e.g., wide use of non-antibiotic compounds in biocides and/or animal feed supplements) and/or adaptive traits (e.g., biofilm production) for the success of *S.* 4,[5],12:i:- in order to contain its ongoing dissemination.

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Conflict of interest The authors declare that they have no conflict of interest.

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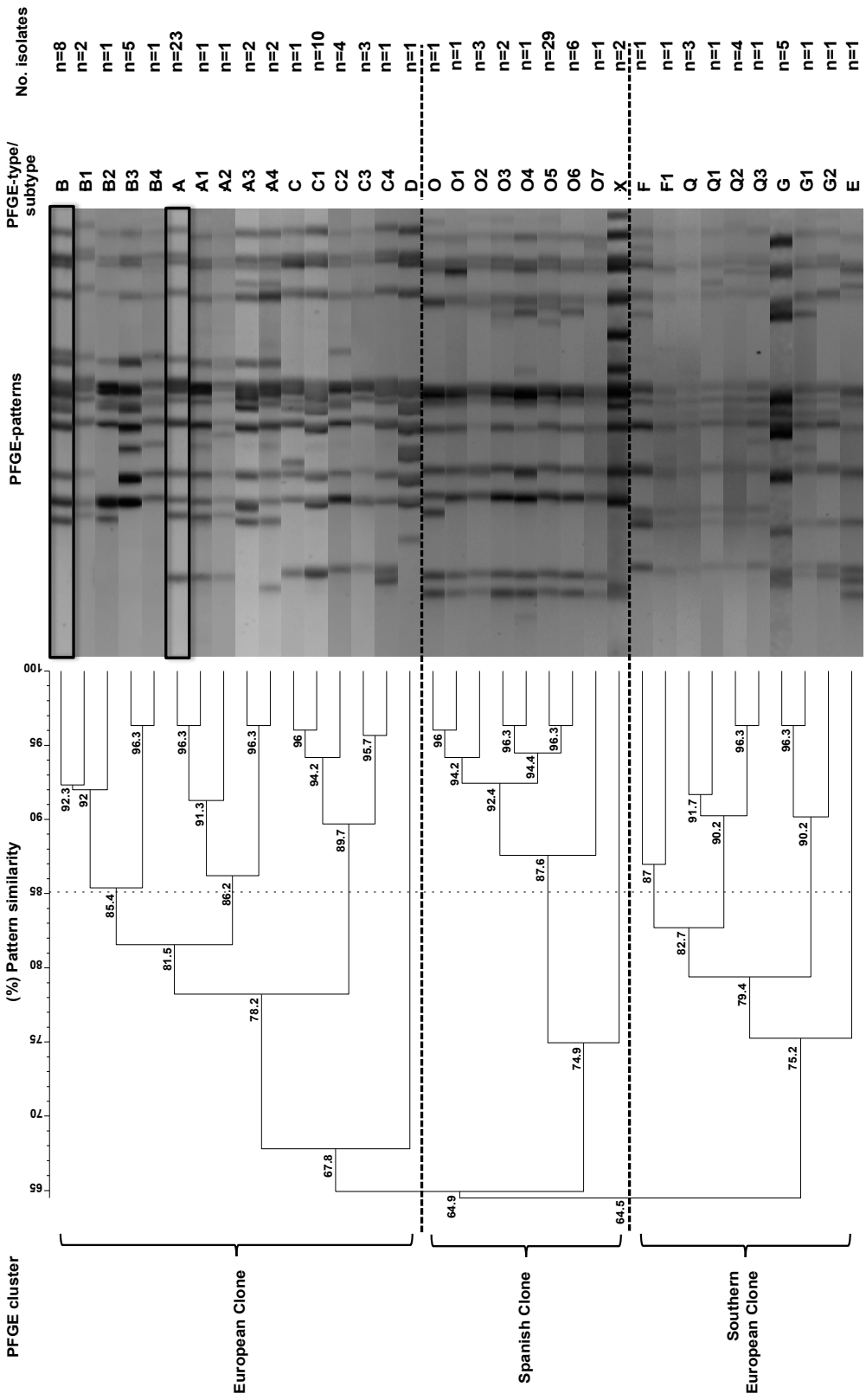

Online Resource 1 *XbaI* profiles of *Salmonella enterica* 4,[5],12:i:- isolates were analysed by InfoQuest FP version 5.4 software (BioRad Laboratories), and the percentage of similarity was calculated by applying the unweighted-pair group method using average linkages (UPGMA) algorithm based on the Dice coefficient (1.0% band tolerance; 1.0% optimization). A rectangle highlights the most prominent PFGE profiles in Europe, A and B corresponding to STYMXB.0079 and STYMXB.0131 respectively (designation according to PulseNet Europe)

3.2. Analysis of the dispersion of metals/biocides tolerance genes and tolerance phenotype as well as their associated genetic platforms in diverse non-typhoidal Salmonella serotypes and clones

3.2.1. Metal tolerance in emerging clinically relevant multidrug-resistant *Salmonella enterica* serotype 4,[5],12:i:– clones circulating in Europe.

3.2.2. Tolerance to multiple metal stressors in emerging non-typhoidal MDR *Salmonella* serotypes: a relevant role for copper in anaerobic conditions.

3.2.3. *Salmonella enterica* serotype Bovismorbificans, a new host for CTX-M-9.

3.2.4. Clinical *Salmonella* Typhimurium ST34 with metal tolerance genes and an IncHI2 plasmid carrying *oqxAB-aac(6')-Ib-cr* from Europe.

Metal tolerance in emerging clinically relevant multidrug-resistant *Salmonella enterica* serotype 4,[5],12:i:- clones circulating in Europe

Joana Mourão[1], Carla Novais[1], Jorge Machado[2], Luísa Peixe[1] and Patrícia Antunes[1,3]*

[1] UCIBIO/REQUIMTE, Departamento de Ciências Biológicas, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal; [2] Laboratório Nacional de Referência de Infeções Gastrointestinais, Departamento de Doenças Infeciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisboa, Portugal; [3] Faculdade de Ciências da Nutrição e Alimentação, Universidade do Porto, Porto, Portugal.

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Metal tolerance in emerging clinically relevant multidrug-resistant *Salmonella enterica* serotype 4,[5],12:i:– clones circulating in Europe

Joana Mourão[a], Carla Novais[a], Jorge Machado[b], Luísa Peixe[a], Patrícia Antunes[a,c,*]

[a] *UCIBIO/REQUIMTE, Departamento de Ciências Biológicas, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal*
[b] *Laboratório Nacional de Referência de Infecções Gastrointestinais, Departamento de Doenças Infecciosas, Instituto Nacional de Saúde Dr Ricardo Jorge, Lisbon, Portugal*
[c] *Faculdade de Ciências da Nutrição e Alimentação, Universidade do Porto, Porto, Portugal*

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ABSTRACT

The occurrence of acquired metal tolerance genes in emerging MDR *Salmonella enterica* serotype 4,[5],12:i:– clones was assessed and their associated platforms and tolerance phenotype were characterised. *Salmonella* 4,[5],12:i:– from different sources belonging to European, Spanish and Southern European clones were studied. Screening for copper (*pcoA-pcoD*/*tcrB*), silver/copper (*silA-silE*), mercury (*merA*), arsenic (*arsB*) and tellurite (*terF*) tolerance genes was performed by PCR/sequencing. $CuSO_4$/$AgNO_3$ MICs were determined in aerobic/anaerobic atmospheres by agar dilution. Conjugation assays, genomic location and plasmid analysis were performed by standard procedures. Most isolates from European (98%) and Spanish (74%) clones carried *silA-silE*, contrasting with the Southern European clone (26%). *merA*/62% (European and Spanish clones) and *pcoA-pcoD*/50% (European clone) were also detected. *merA* ± *pco* + *sil* were chromosomally located in the European clone, whereas in Spanish and Southern European clones *sil* ± *merA* were within plasmids, both with antibiotic resistance genes. The *pcoA-pcoD*/*silA-silE*$^+$ isolates showed higher MIC_{CuSO4} in anaerobiosis than those without these genes (MIC_{50} = 24–28 vs. 2 mM). Different MIC_{AgNO3} of *silA-silE*$^+$ (MIC_{50} = 0.25 mM) and *silA-silE*$^-$ (MIC_{50} = 0.16 mM) isolates were observed in both atmospheres, with an MIC increment after prior exposure to silver (>3 vs. 0.08–0.125 mM) in aerobiosis. A high frequency of copper and silver tolerance, particularly among the two major *Salmonella* 4,[5],12:i:– MDR clones (European/Spanish) circulating in Europe and causing human infections, might facilitate adaptation/expansion of these strains in metal-contaminated environments, particularly copper in anaerobiosis. Furthermore, metal toxic concentrations in food-animal environments can contribute to persistence of genetic platforms carrying metal/antibiotic resistance genes in this foodborne zoonotic pathogen.

1. Introduction

Salmonella enterica subsp. *enterica* serotype 4,[5],12:i:–, a monophasic variant of pathogenic *S. enterica* subsp. *enterica* serotype Typhimurium, has become a new epidemic multidrug-resistant (MDR) serotype in Europe associated with human infections [1,2]. Currently, two major clones are recognised and were previously designated as 'European' and 'Spanish'. They are spread in diverse geographical regions of Europe particularly since 2002 [2,3] or are mainly located in the Iberian Peninsula, respectively [4,5]. A third less frequent clone, the Southern European clone, was recently described in Portugal [5] and was also found among a few sporadic Italian and Spanish isolates [4]. All of these clones, carrying a combination of antibiotic resistance molecular features, account for *Salmonella* 4,[5],12:i:– spread through the food chain, with food animals (e.g. pigs) indicated as their reservoir [2–5]. Selective pressure due to massive use of antibiotics in food-producing animals has been implicated in their selection and maintenance, but the possibility of co-selection of these successful MDR *Salmonella* clones by other compounds widely used in animal settings (e.g. biocides, metals) remains less explored.

Non-antibiotic compounds with antimicrobial activity are currently used in animal farming management (biosecurity, hygiene) owing to antibiotic restriction directives and/or to limit foodborne zoonotic pathogenic bacteria such as

* Corresponding author. Present address: Faculdade de Ciências da Nutrição e Alimentação, Universidade do Porto, Rua Dr Roberto Frias, 4200-465 Porto, Portugal. Tel.: +351 22 507 4320; fax: +351 22 507 4329.
E-mail addresses: patriciaantunes@fcna.up.pt, patantunes@gmail.com (P. Antunes).

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Table 1
Epidemiological and antibiotic and metal tolerance genetic features of *Salmonella enterica* subsp. *enterica* serotype 4,[5],12:i:– isolates from Portugal.

Clone designation Phage type (no. of isolates/ST-eBG)[a] Predominant antibiotic resistance phenotype (no. of isolates) and genotype	PFGE type (no. of isolates; no. of PFGE subtypes)[b]	Source (no. of isolates)	Year(s)/region(s)[c]	Occurrence of metal tolerance genes [no. of isolates (%)]			Metal tolerance genes; location (kb, Inc) (no. of isolates)	Co-location with antibiotic resistance genes (no. of isolates)
				pcoA–pcoD	*silA–silE*	*merA*		
European clone Non DT104/U302 (*n* = 66/ST34-1) AMX, STR, SUL, TET (*n* = 51) bla_{TEM-1}, *strA–strB*, *sul2*, *tet*(B)	A (*n* = 29; 5)	Human (*n* = 26; 16 hospitals, 2 community labs) Environment (*n* = 1; drinking water) Piggeries (*n* = 2; manure, animal feed)	2003–2010/N, C, S	29 (100%)	29 (100%)	11 (38%)	*pcoD* + *silA* + *merA*; Chr[d] (*n* = 4) *pcoD* + *silA*; Chr (*n* = 3)	bla_{TEM-1} and/or *strA–strB*, *sul2*, *tet*(B) (*n* = 7)
	B (*n* = 17; 5)	Human (*n* = 17; 10 hospitals)	2008–2010/N, C, S, I	17 (100%)	17 (100%)	14 (82%)	*pcoD* + *silA* + *merA*; Chr[d] (*n* = 9) *pcoD* + *silA*; Chr (*n* = 1)	bla_{TEM-1}, *strA–strB*, *sul2*, *tet*(B) (*n* = 9) *tet*(B) (*n* = 1)
	C (*n* = 19; 5)	Human (*n* = 19; 15 hospitals)	2007–2010/N, C, S, I	18 (95%)	18 (95%)	13 (68%)	*merA* and/or *pcoD* + *silA*; Chr[d] (*n* = 4) *pcoD* + *silA*; Chr (*n* = 1)	*strA–strB*, *sul2*, *tet*(B) and/or bla_{TEM-1} (*n* = 5)
	D (*n* = 1; 1)	Human (*n* = 1; 1 hospital)	2009/N	1 (100%)	1 (100%)	1 (100%)	*pcoD* + *silA* + *merA*; Chr[d] (*n* = 1)	bla_{TEM-1}, *strA–strB*, *sul2*, *tet*(B) (*n* = 1)
	n = 66			*n* = 65 (98%)	*n* = 65 (98%)	*n* = 39 (59%)		
Spanish clone DT104/U302 (*n* = 46/ST19-1) AMX, CHL, GEN, STR, SUL, TET, TMP (*n* = 26) bla_{TEM-1}, *cmlA1*, *aac(3)-IV*, *aadA2–aadA1*, *sul1–sul2–sul3*, *tet*(A), *dfrA12*	O (*n* = 44; 8)	Human (*n* = 31; 20 hospitals) Food (*n* = 8; poultry, pork, beef) Environment (*n* = 3; bathing water) Piggery (*n* = 2; manure)	2002–2010/N, C, S	0 (0%)	32 (73%)	40 (91%)	*silA* + *merA*; PL (170; A/C, N) (*n* = 1)	bla_{TEM-1}, *cmlA1*, *aac(3)-IV*, *aadA2–aadA1*, *sul1–sul2–sul3*, *tet*(A), *dfrA12*[e] (*n* = 1)
							silA + *merA*; PL (110–130; A/C) (*n* = 3)	bla_{TEM-1}, *cmlA1*, *aac(3)-IV*, *aadA2–aadA1*, *sul1–sul2–sul3*, *tet*(A), *dfrA12*[e] (*n* = 3)
							silA + *merA*; PL (110; A/C) (*n* = 1)	*cmlA1*, *aadA2–aadA1*, *sul2–sul3*[e] (*n* = 1)
	X (*n* = 2; 1)	Human (*n* = 2; 1 hospital)	2004/S	0 (0%)	2 (100%)	2 (100%)	*silA* + *merA*; PL (220; A/C) (*n* = 1)	bla_{TEM-1}, *cmlA1*, *aac(3)-IV*, *aadA2–aadA1*, *sul1–sul3*, *tet*(A), *dfrA12*[e] (*n* = 1)
	n = 46			*n* = 0 (0%)	*n* = 34 (74%)	*n* = 42 (91%)		

Table 1 (*Continued*)

Clone designation Phage type (no. of isolates/ST-eBG)[a] Predominant antibiotic resistance phenotype (no. of isolates) and genotype	PFGE type (no. of isolates; no. of PFGE subtypes)[b]	Source (no. of isolates)	Year(s)/region(s)[c]	Occurrence of metal tolerance genes [no. of isolates (%)]			Metal tolerance genes; location (kb, Inc) (no. of isolates)	Co-location with antibiotic resistance genes (no. of isolates)
				pcoA–pcoD	*silA–silE*	*merA*		
Southern European clone Non DT104/U302 (*n* = 19/ST19-1) CHL, STR, SUL, TET, TMP (*n* = 15) *cmlA1*, *aadA2–aadA1*, *sul3*, *tet*(B), *dfrA12*	Q (*n* = 9; 4)	Human (*n* = 8; 8 hospitals) Food (*n* = 1; pork)	2004, 2006–2010/N, C, S	0 (0%)	0 (0%)	0 (0%)	N/A	N/A
	G (*n* = 7; 3)	Human (*n* = 7; 6 hospitals)	2003–2004, 2009–2010/N	0 (0%)	5 (71%)	0 (0%)	*silA*; PL (110–140; R) (*n* = 5)	*cmlA1*, *aadA2–aadA1*, *sul3*, *tet*(B), *dfrA12*[e] (*n* = 5)
	E (*n* = 1; 1)	Human (*n* = 1; 1 hospital)	2007/S	0 (0%)	0 (0%)	0 (0%)	N/A	N/A
	F (*n* = 2; 2)	Human (*n* = 2; 2 hospitals)	2008–2009/N	0 (0%)	0 (0%)	0 (0%)	N/A	N/A
	n = 19			*n* = 0 (0%)	*n* = 5 (26%)	*n* = 0 (0%)		

ST, sequence type; eBG, eBurstGroups identified by multilocus sequence typing (http://mlst.ucc.ie/mlst/dbs/Senterica); PFGE, pulsed-field gel electrophoresis; AMX, amoxicillin; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; CHL, chloramphenicol; GEN, gentamicin; TMP, trimethoprim; Chr, chromosome; PL, plasmid; N/A, not applicable.

[a] PCR assay for identification of *Salmonella enterica* serotype Typhimurium DT104/U302 as previously described [5].

[b] Clones were designated by capital letters and also include PFGE subtypes as previously described [5].

[c] N, North; C, Centre; S, South; I, Islands (Azores).

[d] Co-location in the same I-*CeuI* chromosomal fragment as antibiotic resistance genes was observed for all isolates with the *merA* gene (*n* = 18), including 5 isolates also carrying *pcoD*+*silA* (PFGE types B and D).

[e] Some genes belong to the atypical *sul3*-type III integron (5′CS-*estX–psp–aadA2–cmlA1–aadA1–qacH*–IS*440–sul3*) and/or the conventional class 1 integron (*dfrA12–orfF–aadA2–cmlA1–aadA1–qacH*–IS*440–sul3*) in the Southern European clone [5].

Salmonella (including *Salmonella* 4,[5],12:i:−) in the food supply (http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32011R0517&from=EN) [6,7]. Several metals are suitable for use as disinfectants, antiseptics or preservatives (e.g. silver, copper) and as feed additives for growth promotion of farm animals (e.g. copper, zinc) (http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32003R1831&from=EN) or can be found as contaminants in animal feed (e.g. mercury) (http://www.efsa.europa.eu/en/efsajournal/doc/654.pdf) [6,7]. Metals can remain in the production environment and accumulate in toxic concentrations, representing a long-term selective pressure potentially driving co-selection of antibiotic-resistant bacteria, including *Salmonella* [6–8]. Diverse acquired genes encoding for metal tolerance mechanisms have been described in scarce studies of *Salmonella* and other Gram-negative bacteria (e.g. Enterobacteriaceae, *Aeromonas*, *Campylobacter*, *Pseudomonas*) commonly sharing the same ecosystems [9–15]. Among them are efflux systems to overcome toxic concentrations of copper (e.g. *pcoABCDRSE* and *tcrYAZB*), silver (e.g. *silCFBAPRSE*) or arsenic (e.g. *arsRBC*); mercuric detoxification systems (e.g. *mer* operons); and the tellurite resistance system (e.g. *terZABCDEF*). Nevertheless, a comprehensive analysis of metal tolerance, including acquired metal gene dispersion and their genetic context within *Salmonella* from diverse ecological niches or belonging to successful MDR clones, remains poorly explored.

This study assessed the occurrence of acquired metal tolerance genes in emerging MDR *Salmonella* 4,[5],12:i:− clones and characterised their associated genetic platforms and tolerance phenotype.

2. Materials and methods

2.1. Strains and epidemiological background

This study included a comprehensive collection of 131 *Salmonella* 4,[5],12:i:− isolates obtained between 2002 and 2010 from human clinical cases (*n* = 114 from 28 hospitals and 2 community laboratories) covering all geographical regions of Portugal (North, Centre, South and Azores islands) as well as from food of animal origin (*n* = 9; pork, beef and poultry), the environment (*n* = 4; drinking and bathing water) and piggeries (*n* = 4; manure and animal feed) (Table 1). Clonality by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST), MDR profiles, *fljAB* deletion patterns and genetic elements linked to antibiotic resistance were previously characterised for the three discriminated clones (European, Spanish and Southern European) [5]. The occurrence of diverse acquired metal tolerance genes was assessed in all of the isolates from this collection. Representative isolates from different clonal lineages, PFGE types, sources, isolation date and antibiotic resistance phenotypes and genotypes, harbouring or lacking acquired copper (Cu)/silver (Ag) tolerance genes, were selected for phenotypic assays [minimum inhibitory concentration (MIC) determination] and for characterisation of genetic platforms.

2.2. Study of metal tolerance and acquired genes

Different acquired genes previously described among Gram-negative and Gram-positive bacteria and associated with tolerance to toxic concentrations of metals, widely used or present in animal settings, were searched by PCR using the conditions and primers described in Supplementary Table S1. They include genes from different metal export systems, namely two genes of the *pcoABCDRSE* cluster [*pcoA* (multicopper oxidase) and *pcoD* (copper inner membrane pump)] [9,16], one gene of the *tcrYAZB* cluster [*tcrB* (copper export ATPase)] [17], two genes of the *silCFBAPRSE*

cluster [*silA* (silver inner-membrane proton/cation antiporter) and *silE* (silver/copper periplasmic metal binding protein)] [18] and *arsB* (arsenite transmembrane pump) [11]. Other mechanisms related to metal tolerance, such as *merA* (mercuric reductase) [10] and *terF* (tellurite resistance protein), were also included [11]. A positive amplicon from each PCR was sequenced and compared with known GenBank sequences to validate the screening method (Supplementary Table S1).

Supplementary Table S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijantimicag.2015.01.013.

MICs were determined by the agar dilution method using Mueller–Hinton II agar plates (bioMérieux, Marcy-l'Étoile, France) supplemented with $CuSO_4$ (0.25, 0.5, 1, 2, 4, 8, 12, 16, 20, 24, 28, 32 and 36 mM, adjusted to pH 7.2) or $AgNO_3$ (0.025, 0.06, 0.08, 0.125, 0.16, 0.25, 0.32, 0.5, 1.0 and 1.5 mM, adjusted to pH 7.4) as described previously [19]. Briefly, 1 μL of a 10^7 CFU/mL suspension of each isolate was applied to the supplemented plates, which were incubated at 37 °C in aerobic and anaerobic (GENbox jar with GENbox anaer. and an anaerobic indicator; bioMérieux) atmospheres and read at 16–20 h. The first $CuSO_4$/$AgNO_3$ concentration without visible bacterial growth was considered as the MIC. Since *sil* genes have been described as inducible by silver in an aerobic atmosphere [20], MICs to $AgNO_3$ were also determined following overnight *sil*$^+$ and *sil*$^-$ cultures (14–16 h) in BBL™ Mueller–Hinton II broth (BD, Franklin Lakes, NJ) supplemented with 25 μM of this metal. *Enterococcus faecium* BM4105RF [17] or *Staphylococcus aureus* ATCC 29213 [21] (both negative for all genes tested) and *Escherichia coli* ED8739 (plasmid pRJ1004 with *pco* genes) [9] or *E. coli* J53 (plasmid pMG101 with *sil* genes) [21] were used as controls in $CuSO_4$ or $AgNO_3$ tolerance assays, respectively.

2.3. Characterisation of the genetic elements carrying metal and antibiotic resistance genes

Conjugative transfer of antibiotic resistance and metal tolerance genes was analysed for 20 representative isolates of different clonal lineages, PFGE types, resistance phenotypes/genotypes and sources. Conjugative transfer was performed by the filter-mating method on Mueller–Hinton II agar plates at 1:1 and 1:2 donor:recipient ratios using *E. coli* K802N or *E. coli* K-12 strain BM21R as recipient strains, respectively (both rifampicin- and nalidixic acid-resistant) at 30 °C, 37 °C and 42 °C. Transconjugants were recovered after incubation at 37 °C from Mueller–Hinton II agar or MacConkey agar plates supplemented with nalidixic acid (64 mg/L) plus tetracycline (8 mg/L) or sulfamethoxazole (256 mg/L). Co-transfer of antibiotic resistance and/or metal tolerance was confirmed at phenotypic and genotypic levels as described above. Identification of plasmid incompatibility groups was determined by the PCR-based replicon typing (PBRT) method, including three additional PCR assays for the IncU, IncR and ColE groups as previously described [5]. The genetic location of metal and antibiotic determinants was determined by S1 nuclease- (Takara Bio Inc., Shiga, Japan) and I-*Ceu*I- (New England Biolabs, Ipswich, MA) digested genomic DNA followed by PFGE and Southern blot hybridisation. This was performed by standard methods using antibiotic [*aac(3)-IV*, bla_{TEM-1}, *strA*, *strB*, *sul1*, *sul2*, *sul3*, *tet*(A), *tet*(B), *dfrA12*] and metal (*pcoD*, *silA*, *merA*, *arsB*, *terF*) resistance genes, *intI1*, rep intragenic probes (IncA/C, R) and 16S rRNA following the manufacturer's instructions (Gene Images AlkPhos Direct™ Labelling System; Amersham GB/GE Healthcare Life Sciences UK Ltd., Little Chalfont, UK). *Xba*I-digested *S. enterica* serotype Braenderup H9812 (CDC) was used as the reference strain.

3. Results

3.1. Occurrence of acquired genes encoding metal tolerance

The genes *silA–silE*, *merA* and *pcoA–pcoD* were detected in 79% (104/131), 62% (81/131) and 50% (65/131) of the isolates, respectively. The *silA–silE* genes were dispersed among the majority of isolates belonging to the European (98%; all PFGE types) and Spanish (74%; all PFGE types) clones and in a few belonging to the Southern European clone (26%; only PFGE type G), regardless of their source (human, environment, piggeries and food) or resistance profile. In contrast, the *pcoA–pcoD* genes were only detected in *silA–silE*$^+$ isolates from the European clone (98%; $n = 65$) (Table 1). Combinations of *sil* + *merA* (34/42) or *pco* + *sil* + *merA* (38/39) were frequently found among Spanish and European clones, respectively. None of the other searched genes was detected.

3.2. Genetic elements carrying metal and antibiotic resistance genes

Isolates of the European clone presented *pco* + *sil* and/or *merA* in the chromosome. In addition, these metal tolerance genes were often co-located in the same chromosomal I-*Ceu*I PFGE fragment of ca. 750 kb and/or ca. 850 kb as antibiotic resistance genes typical of this clonal lineage [bla_{TEM-1}, *strA–strB*, *sul2* and *tet*(B)], none of them being transferable by conjugation assays (Table 1). In contrast, Spanish clone isolates carried *sil* + *merA* within ca. 110–220 kb non-transferable IncA/C plasmids, along with a *sul3*-type III class 1 integron (*estX–psp–aadA2–cmlA1–aadA1–*IS*440–qacH–sul3*), bla_{TEM-1}, *aac(3)-IV*, *tet*(A), *dfrA12*, *sul1–sul2*, typical of this clonal lineage (Table 1). In the Southern European clone, *sil* was found co-located in ca. 110–140 kb non-transferable IncR plasmids with *tet*(B) and an atypical *sul3*-type I class 1 integron (*dfrA12–orfF–aadA2–cmlA1–aadA1–*IS*440–qacH–sul3*) genes, only in isolates from PFGE type G (Table 1).

3.3. Susceptibility to $CuSO_4$ and $AgNO_3$ in aerobic and anaerobic atmospheres

The MIC_{CuSO4} distribution was similar for isolates of the three clones, sources and independently of location (chromosome or plasmid) of metal tolerance determinants. Higher MIC_{CuSO4} values were obtained in aerobic conditions (MIC_{50} = 32–36 mM) irrespective of the presence or absence of *pcoA–pcoD* and/or *silA–silE*. However, in an anaerobic atmosphere, the *pcoA–pcoD*/*silA–silE*$^+$ isolates showed higher MIC_{CuSO4} values than those without these genes (MIC_{50} = 24–28 mM vs. 2 mM) (Table 2). Regarding $AgNO_3$, the MIC_{50} was identical both in aerobic and anaerobic conditions, but differences between isolates with *silA–silE* (MIC_{50} = 0.25 mM) and without *silA–silE* (MIC_{50} = 0.16 mM) in the two atmospheres were observed. In addition, in isolates that were subjected to prior exposure to subinhibitory concentrations of $AgNO_3$, MICs for those carrying *silA–silE* increased to >3 mM, contrasting with *silA–silE*$^-$ isolates (0.08–0.125 mM) (Table 2).

4. Discussion

This is the first study documenting a high occurrence of genes encoding tolerance to copper and silver, particularly dispersed among the two major *Salmonella* 4,[5],12:i:– MDR clonal lineages (European and Spanish clones) circulating in Europe and causing human infections. These features might have a potential role in the adaptation and expansion of these strains in metal-contaminated environments.

An association between silver tolerance and presence of the *sil* efflux system among isolates belonging to *Salmonella* epidemic

Table 2
Minimum inhibitory concentrations (MICs) of $CuSO_4$ and $AgNO_3$ for *Salmonella enterica* subsp. *enterica* serotype 4,[5],12:i:– isolates.

Metal	Environment	*silA–silE*	*pcoA–pcoD*	No. of isolates[a]	MIC (mM)[b]														MIC$_{50}$ (mM)	MIC$_{90}$ (mM)
					0.08	0.125	0.16	0.25	0.32	0.5	1	2	>3	20	24	28	32	36		
$CuSO_4$	Aerobic	+	+	30												12	14	4	32	36
		+	–	19												1	8	10	36	36
		–	–	15												7	7	1	32	32
	Anaerobic	+	+	30										7	12	10	1		24	28
		+	–	19										1	7	9	1	1	28	32
		–	–	15															2	2
											1	5	9							
$AgNO_3$	Aerobic	+		48		1	9	7	7	23	1								0.25	0.32
		–		16			3	13											0.16	0.16
	Anaerobic	+		48			2	7	27	4	8								0.25	0.5
		–		16				8	7	1									0.16	0.25
	Aerobic/previous contact with 0.025 mM $AgNO_3$	+		16									16						>3	>3
		–		8		3	5												0.125	0.125

[a] The number of isolates included representatives of the three clones: European clone ($n = 31$); Spanish clone ($n = 19$); and Southern European clone ($n = 14$).
[b] MIC values of $CuSO_4$ (4, 8, 12 and 16 mM) and $AgNO_3$ (0.75 and 1.5 mM) that were not assigned to the isolates tested were omitted. *Escherichia coli* ED8739 (plasmid pRJ1004 with *pco* genes; MIC_{CuSO4} = 32–36 mM, aerobiosis) [9] and *Enterococcus faecium* BM4105RF (negative for all genes tested; MIC_{CuSO4} = 8–12 mM, aerobiosis) [17] were used as control strains in Cu assays; Those strains were also used in anaerobic assays: *E. coli* ED8739 (plasmid pRJ1004 with *pco* genes; MIC_{CuSO4} = 16–20 mM) and *E. faecium* BM4105RF (MIC_{CuSO4} = 2–4 mM). *Escherichia coli* J53 (plasmid pMG101 with *sil* genes; MIC_{AgNO3} > 3 mM, aerobiosis) [21] and *Staphylococcus aureus* ATCC 29213 (negative for all genes tested; MIC_{AgNO3} = 0.16–0.25 mM, aerobiosis) [21] were used as control strains in Ag assays. Those strains were also used in anaerobic assays: *E. coli* J53 (plasmid pMG101 with *sil* genes; MIC_{AgNO3} > 3 mM) and *S. aureus* ATCC 29213 (MIC_{AgNO3} = 0.32–0.5 mM).

clones was established in this study. Although *sil* genes are scattered in different bacterial genera [12–14,18], including diverse *Salmonella* serotypes (e.g. GenBank accession nos. AF067954, CP005995, CP006055 and CP007505), an association between the genotype and the corresponding phenotypic behaviour has never been established in a comprehensive *Salmonella* collection. In addition, scarce studies searching for *sil* tolerance genes are confined to non-*Salmonella* Enterobacteriaceae isolates recovered from nosocomial and animal clinical settings [12–14] or environmental bacteria [22], impairing an evaluation of *sil* tolerance gene dispersion and their potential impact on the success of emergent *Salmonella* in particular environments, namely where silver could be widely used (e.g. disinfectants used in water or surfaces in the animal production setting; human and animal antiseptics) [22,23].

Higher tolerance to $CuSO_4$ in anaerobic conditions for *Salmonella* 4,[5],12:i:– isolates carrying *sil* or *sil* + *pco* genes was detected, suggesting that they could overcome the highly toxic cuprous form (Cu^{1+}) present in anoxic environments [24] (e.g. animal gastrointestinal tract and manure, waste lagoons, or forage/feed from food-producing settings). It was proposed that the *silE* gene may have a role in copper tolerance under anaerobic conditions [18], contrasting with other acquired aerobic copper tolerance gene clusters such as the plasmid-borne *pcoABCDRSE* efflux system (e.g. *pcoA* requires oxygen to convert Cu^{1+} into the less toxic Cu^{2+}) in *E. coli* [9,16]. However, similar to another recent study [15], the high MIC_{CuSO4} detected in aerobic conditions, both in *Salmonella* carrying *pco* or not, indicate that these genes might not lead to an increase in copper tolerance in *Salmonella* 4,[5],12:i:– isolates in such environmental conditions. The common use of copper as a feed additive in animal growth promotion is a current concern since it might contribute to the selection and maintenance of copper-tolerant bacteria (http://www.efsa.europa.eu/en/efsajournal/doc/2969.pdf) [25]. In fact, the emergence of *Salmonella* 4,[5],12:i:– clones causing human infections has been associated particularly with pig production [1–3,26], which are within the animal settings where higher copper concentrations in feed are allowed (http://eur-lex.europa.eu/LexUriServ/%20LexUriServ.do?uri=OJ:C:2004:050:0001:0144:EN:PDF) by the European Union.

Co-localisation of the metal tolerance genes within chromosomal regions or plasmids previously associated with MDR profiles in *Salmonella* 4,[5],12:i:– clones [4,5,27], irrespective of their source, was shown by the molecular studies performed. In fact, it is widely demonstrated in industrialised countries that *Salmonella* MDR genetic platforms are the same both in food-production animals and humans, suggesting a direct link between the two bacterial populations. In the case of the globally spread European *Salmonella* 4,[5],12:i:– clone, *mer* genes were recently found downstream of the RR1–RR2 resistance region [27], similar to *Salmonella* genomic island 1 (SGI-1) variants [28], but genetic data pointing out the potential to adapt simultaneously to other metal environmental stresses (e.g. copper and silver) is described here first. The chromosomal co-localisation of *pco* + *sil* and *mer*, frequently associated with antibiotic resistance genes, appears to be typical of the European clone. These data, along with the recently reported chromosomal integration of Tn*7*-like transposons carrying *pco* + *sil* in an *S. enterica* serotype Senftenberg [29], suggests that the accumulation of diverse metal tolerance genetic determinants with other features might relevantly contribute to the evolution or emergence of global widespread emergent clones. Although several studies related the emergence and/or spread of Spanish and Southern European clones to the presence of IncA/C or IncR plasmids harbouring additional MDR features and virulence factors [4,5,26], this study also suggests a role for metal tolerance genes. In fact, *sil* + *merA* and antibiotic resistance genes were linked together on the IncA/C plasmid typical of the second major clone (Spanish clone) circulating

in Europe, contrasting with the less frequent clone (Southern European clone) where the presence of *sil* genes occurred in few isolates associated with IncR MDR plasmids. Metal tolerance genes are dispersed in diverse plasmid backbones (e.g. *merA* in IncA/C or *sil* in IncHI) [11,20,29,30] within different bacterial hosts potentially facing similar chemical challenges, which might favour the sharing and persistence of these genetic elements in particular environments [30]. The ability to acquire diverse genetic platforms with genes coding for a phenotype plethora (e.g. antibiotic resistance, metal tolerance, virulence) might facilitate adaptation of zoonotic clinically relevant strains or epidemic clones such as *Salmonella* 4,[5],12:i:–.

5. Conclusions

In summary, we report a high occurrence of genes encoding tolerance to toxic concentrations of $CuSO_4$ and $AgNO_3$ dispersed among the two major *Salmonella* 4,[5],12:i:– MDR clonal lineages (European and Spanish clones) circulating in Europe and causing human infections, which might facilitate the adaptation/expansion of these strains in metal-contaminated environments, particularly copper in anoxic conditions. Furthermore, metal toxic concentrations in the food-animal farm environment can contribute to the persistence of genetic platforms carrying metal and antibiotic resistance genes in this clinically relevant foodborne zoonotic pathogen. More studies are needed to evaluate the impact of the continuous contact with subinhibitory concentrations of several metal environmental contaminants from feed, disinfectants and/or other anthropogenic sources, and their impact on the dynamics of the emergence of different *Salmonella* serotypes and clones.

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Competing interests

None declared.

Ethical approval

Not required.

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Supplementary Table S1
Primers used in the PCR to search for acquired metal tolerance genes

Target gene	Gene coding for	Primer	Primer sequence (5'–3')	Amplicon size (bp)	Annealing temperature (ºC)	Nucleotide position (GenBank accession no.)	Reference
pcoA	Multicopper oxidase	pcoA_978_F pcoA_1481_R	CTCGCGGATGTCAGTGGCTACACCT ATCCGGAAGGTCAGCACCGTCCATAGAC	504	60	978–1002 1481–1454 (**X83541.1**)	This study
pcoD	Copper inner membrane pump	pcoD_F pcoD_R	CTGGCCACACTTGCCTGGGG CACGCTACGGCGCCCAGAAT	500	55	3801–3820 4300–4281 (**X83541.1**)	This study
tcrB	Copper export ATPase	tcrB_F tcrB_R	CATCACGGTAGCTTTAAGGAGATTTTC ATAGAGGACTCCGCCACCATTG	663	66	4954–4980 5616–5595 (**AY048044.2**)	[17]
silA	Silver inner-membrane proton/cation antiporter	silA_Fw silA_Rv	GCAAGACCGGTAAAGCAGAG CCTGCCAGTACAGGAACCAT	936	59	8936–8955 9871–9852 (**AF067954.1**)	This study
silE	Silver/copper periplasmic binding protein	silE_1105_F silE_1368_R	GTTCGTCATGGTYTCATGAGC GTACTYCCCCGGACATCACTAATT	264	62	1105–1125 1368–1345 (**AF067954.1**)	This study
merA	Mercuric reductase	merA_1F merA_5R	ACCATCGGCGGCACCTGCGT ACCATCGTCAGGTAGGGGAAC	1238	65	2140–2159 3377–3357 (**K03089.1**)	[10]
arsB	Arsenite transmembrane pump	arsB_Fw arsB_Rv	AGTGAAAGACAGACGAAGCG GGCAGATAGTGTGGAATGCG	1136	60	159735–159754 160870–160851 (**BX664015.1**)	[11]
terF	Tellurite resistance protein	terF_Fw terF_Rv	ATGCAGGCTCAAGGAATCGC TTCATCGATCCACGGTCTG	894	60	80270–80289 81163–81145 (**BX664015.1**)	[11]

Tolerance to multiple metal stressors in emerging non-typhoidal MDR *Salmonella* serotypes: a relevant role for copper in anaerobic conditions

Joana Mourão[1], Sara Marçal[2], Paula Ramos[2], Joana Campos[1], Jorge Machado[3], Luísa Peixe[1], Carla Novais[1] and Patrícia Antunes[1,2]*

[1] UCIBIO/REQUIMTE, Departamento de Ciências Biológicas, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal; [2] Faculdade de Ciências da Nutrição e Alimentação, Universidade do Porto, Porto, Portugal; [3] Laboratório Nacional de Referência de Infeções Gastrointestinais, Departamento de Doenças Infeciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisboa, Portugal.

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***Corresponding author:**
Patrícia Antunes, Ph.D.
Faculdade de Ciências da Nutrição e Alimentação, Universidade do Porto.
Rua Dr. Roberto Frias, 4200-465 Porto, Portugal
E-mail address: patriciaantunes@fcna.up.pt
Tel.: +351 22 507 4320
Fax: +351 22 507 4329

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SYNOPSIS

Objectives: The driving factors for the expansion of particular MDR *Salmonella* serotypes/clones are not completely understood. Here we assessed if emergent MDR *Salmonella* serotypes/clones were more enriched in metal tolerance genes (e.g. to Cu/Ag) than other less frequent ones, as an additional survival adaptive feature to environments contaminated with metals.

Methods: Metal (Cu-*pco*/Ag,Cu-*sil*/Hg-*mer*/As-*ars*/Te-*ter*) tolerance genes screening (PCR/sequencing), MICs to $CuSO_4$/$AgNO_3$ (aerobiosis/anaerobiosis), genetic elements characterisation (S1/I-CeuI PFGE) and conjugation assays were performed in a well-characterised *Salmonella* collection (n=275 isolates; 2000-2014; 49 serotypes/clones).

Results: The *sil*±*pco* genes were detected in 37% of isolates from diverse serotypes, mainly in emergent Rissen/ST469 and Typhimurium/ST34 European clone (100%) which are mostly associated with pig-setting where copper is highly used. These genes were frequently co-located with *merA*±*terF* and/or antibiotic resistance genes in plasmids (100-270kb; IncHI2/IncHI1/IncN/IncFIIA; mostly transferable by conjugation) or in chromosome. Most *sil*±*pco*$^+$ isolates (77%) were MDR contrasting with *sil*±*pco*$^-$ ones (48%). The *sil*±*pco*$^+$ isolates presented significantly higher MIC_{CuSO4} under anaerobiosis (MIC_{50}/MIC_{90}=28/32mM) and MIC_{AgNO3} after previous Ag contact (MIC_{50}/MIC_{90}>3mM) than *sil*$^-$ ones (MIC_{50}/MIC_{90}=2/8mM to CuSO4; MIC_{50}/MIC_{90}=0.125/0.16 to AgNO3). The use of these modified methodological approaches allowed the establishment of tolerance cut-offs to $CuSO_4$/$AgNO_3$, here firstly proposed.

Conclusions: This study demonstrates that Cu/Ag tolerance genes acquisition might account for the emergence of particular clinically relevant MDR *Salmonella* serotypes/clones by facilitating their adaptation to diverse metal contaminated settings, especially to pig production. An assessment of control measures for the use and/or accumulation of metals in diverse environments is needed to prevent a wider expansion of such strains or the emergence of new ones.

INTRODUCTION

Non-typhoidal *Salmonella enterica* is a worldwide major foodborne pathogen with a main reservoir in the intestinal tract of food-producing animals, from which it is readily transmitted to farm surrounding environments and the food chain.[1,2] In recent years, expansion of particularly successful *Salmonella* serotypes and clonal lineages have been reported globally. They are scattered to humans mostly by a wide range of foodstuffs of animal origin, they are involved in endemic/epidemic situations and they frequently show resistance to multiple antibiotics limiting therapeutic options when necessary.[1-5]

The exact mechanisms of successful *Salmonella* serotypes persistence and spread among animals and in the environment still remain largely unknown. However, recent studies pointed out tolerance to antimicrobial non-antibiotic compounds widely used in food-producing animals as a potential adaptive advantage in environments and hosts (animal/human) contaminated with these type of compounds.[4,6] Among them are heavy metals, currently associated with diverse anthropogenic activities as they are incorporated in biocidal products, such as disinfectants, antiseptics/medicines or preservatives (e.g. silver-Ag; copper-Cu), in feed additives for animal growth promotion (e.g. copper, zinc) or in soil fertilisers (e.g. arsenic).[7-11] Consequently, metals as environmental pollutants can accumulate for example in manure, waste lagoons or feed in the animal production setting,[9,10,12] and then be spread by sewage sludge and liquid manure applied in amended soils worldwide or by water bodies.[9,13-15] This extensive environmental metal contamination is a topic of increasing concern recognized by diverse authorities[8,10,16,17] as they can contribute for the selection of metal tolerant and/or antibiotic resistant bacteria by mechanisms of cross or co-resistance.[9,11,18] Metals can also potentially participate as a catalyst of gene transfer among diverse microorganisms sharing the same ecologic niche.[9,17] A recent study from our group showed that *S.* 4,[5],12:i:-, a worldwide emergent MDR serotype, carries diverse metal tolerance genes to handle with metal toxicity, such as those encoding for Cu/Ag efflux (*sil*/*pco* genes) and mercury-Hg enzymatic reduction (*mer* genes).[6] We also showed that the *sil* gene cluster was frequent among the two major *S*. 4,[5],12:i:- clones (European/Spanish) and that its presence was particular relevant for Cu tolerance under anaerobic conditions,[6] as suggested by others.[19,20] Nevertheless, a comprehensive analysis of acquired metal tolerance by additional *Salmonella* serotypes remains scarce, including for other emergent ones.

Here we hypothesise that emergent antibiotic resistant *Salmonella* serotypes/clones are more enriched in metal tolerance genes (e.g. to Cu and Ag) than other less frequent ones, and that this is an additional survival adaptive feature

contributing to their emergence in metal contaminated environments. To test this hypothesis, we used a large Portuguese *Salmonella* collection representative of different serotypes/clones, in order to search and characterise diverse metal tolerance genes as well as their associated genetic platforms and tolerance phenotype.

MATERIALS AND METHODS

Bacterial isolates and epidemiological background

From a collection of 2500 Portuguese non-typhoidal *Salmonella enterica*, 275 isolates belonging to 49 serotypes were selected based on source, antibiotic susceptibility phenotypes and genotypes (MDR was considered when resistance to ≥ 3 antibiotics of different families was observed), PFGE types and/or Sequence types (ST) obtained by MLST (Table 1 and Table S1, available as Supplementary data at JAC Online).[1,21-25] They were collected between 2000-2014 in several geographical regions of Portugal (North, Centre, South, Islands) from human clinical cases [biological products from hospitals (n=108; articular liquid, blood, faeces, peritoneal fluid, pus or urine) and community laboratories (n=11; faeces)], food products (n=89; beef, cow, pork, poultry, quail, cooked meal), food-animal production setting (n=50-pigs/piggeries environment; n=4-aquacultures environment) and the aquatic environment (n=13; drinking, bathing or river water). These isolates belonged to the two most frequent serotypes worldwide [*Salmonella* Enteritidis (n=32/ST11) and *Salmonella* Typhimurium (n=96/ST19 and ST34)], but also to others that are emergent and have clinical relevance [e.g. *Salmonella* Rissen (n=50/ST469), *Salmonella* Derby (n=16), *Salmonella* Hadar (n=4), *Salmonella* Heidelberg (n=2), *Salmonella* Infantis (n=3), *Salmonella* Newport (n=1), *Salmonella* Saintpaul (n=3), *Salmonella* Stanley (n=2), *Salmonella* Virchow (n=4)]. Thirty-eight less frequently serotypes detected in surveillance studies were also included (Table 1 and Table S1, available as Supplementary data at JAC Online).

Search of acquired metal tolerance genes and copper/silver susceptibility

The occurrence of diverse acquired metal tolerance genes was assessed in all isolates (n=275) and was performed by PCR using conditions and primers previously described for the *S.* 4,[5],12:i:- collection.[6] The searched genes encoded different metal tolerance mechanisms (e.g. export, reduction) related to copper, silver, mercury, arsenite and tellurite: two genes of *pcoABCDRSE* cluster, the *pcoA* (multicopper oxidase) and *pcoD* (copper inner membrane pump); two genes of *silCFBAPRSE* cluster, the *silA* (silver inner-membrane proton/cation antiporter) and *silE* (silver/copper periplasmic metal binding protein); *arsB* (arsenite transmembrane pump); *merA* (mercuric reductase) and

terF (tellurite resistance protein) (Table S2, available as Supplementary data at JAC Online). Representative isolates selected based on presence (n=38) or absence (n=18) of *silA*-*silE* or *pcoA*-*pcoD* genes, anaerobic MIC_{CuSO4} values, serotype, PFGE-type and antibiotic resistance profiles, were further characterised for the presence or absence of *silCPRS* and/or *pcoRSE* gene clusters. Primers and conditions were described in Table S2, available as Supplementary data at JAC Online.

Representative isolates (n=166) from all serotypes and different clonal lineages, PFGE-types, sources, isolation date and antibiotic resistance phenotypes and genotypes, harbouring or lacking acquired copper/silver tolerance genes, were selected for phenotypic assays. MICs were determined in aerobic and anaerobic (GENbox jar with GENbox anaer. and an anaerobic indicator; bioMérieux) atmospheres by the agar dilution method on Mueller-Hinton II agar plates (bioMérieux) supplemented with $CuSO_4$ (0.25, 0.5, 1, 2, 4, 8, 12, 16, 20, 24, 28, 32, 36 mM; adjusted to pH=7.2) or $AgNO_3$ (0.025, 0.06, 0.08, 0.125, 0.16, 0.25, 0.32, 0.5, 0.75, 1.0, 1.5, 3 mM; adjusted to pH=7.4), as described.[6] MICs to $AgNO_3$ were also determined after overnight incubation of *sil*$^+$ and *sil*$^-$ cultures (14-16h) in Mueller-Hinton II broth (BBL, BD, Franklin Lakes, NJ, USA) supplemented with 0.025 mM of this metal for induction of *silCFBAPRSE* cluster.[6] Two plates (the first and last to be inoculated) of Mueller-Hinton II agar without $CuSO_4$ or $AgNO_3$ were included in all MIC assays to assess growth ability of every isolates in different atmospheres. *Enterococcus faecium* BM4105RF[26] or *Staphylococcus aureus* ATCC 29213[27] (both negative for all genes tested) and *Escherichia coli* ED8739[28] (plasmid pRJ1004 with *pco* genes) or *E. coli* J53[27] (plasmid pMG101 with *sil* genes) were used as controls in $CuSO_4$ or $AgNO_3$ tolerance assays.

Characterisation of the genetic platforms carrying metal tolerance genes

Characterisation of genetic platforms (genomic location and plasmid analysis) was carried out in isolates positive for metal tolerance genes, selected accordingly to the serotype and/or PFGE-type frequency. Plasmid analysis was based on identification of incompatibility groups by PCR-based replicon typing (PBRT) method[29,30] and sequencing. The genetic location of metal and antibiotic resistance determinants in plasmids or chromosome was accomplished by Southern blot hybridisation of S1 nuclease (Takara Bio Inc., Shiga, Japan) and I-*Ceu*I (New England BioLabs, Ipswich, MA, USA) digested genomic DNA with intragenic metal tolerance (*pcoD*, *silA*, *merA*, *arsB*, *terF*) and antibiotic resistance genes, *int*I1, plasmid incompatibility groups and 16S rRNA probes, by standard methods.[6] In order to analyse the dispersion of *sil* and *pco* metal tolerance genes and their genetic context in *Salmonella* from other

serotypes and diverse epidemiological scenarios, a detailed GenBank *in silico* analysis in available genomes until October 2015 was also performed (Table S3, available as Supplementary data at JAC Online).

Conjugation assays

Transfer of genetic elements carrying metal tolerance and/or antibiotic resistance genes was assessed by standard conjugation assays.[6] Transfer of copper/silver tolerance was analysed for isolates carrying *sil* and/or *pco* or lacking known genes, but exhibiting high MIC values to $CuSO_4$ (≥24 mM) in anaerobiosis. It was performed by filter-mating method with log phase growth cells at 1:1 donor:recipient ratio, at 37ºC to known recipient strains (*Escherichia coli* K802N and *S.* Typhimurium LSP 448/02, with antibiotic and metal susceptibility shown in Table 4). Transconjugants obtained from isolates with *sil*±*pco* genes were recovered after incubation at 37ºC/24h from Mueller-Hinton II agar plates supplemented with nalidixic acid (64 mg/L) plus other antibiotics [ampicillin (16 mg/L), streptomycin (32 mg/L), sulfamethoxazole (256 mg/L) or trimethoprim (8 mg/L)] in aerobic conditions. Transconjugants obtained from isolates without *sil*±pco genes but with high MIC_{CuSO4} in anaerobic conditions were recovered in Mueller-Hinton II agar plates supplemented with $CuSO_4$ (8 mM or 12 mM, according to the recipient strain used). Confirmation of transconjugants, tolerance to Cu/Ag and co-transfer of antibiotic resistance was confirmed at phenotypic and genotypic levels as previously described.[5,6]

RESULTS

Acquired metal tolerance genes were dispersed in diverse Salmonella serotypes, especially clinical relevant MDR pig-associated clones

The Cu and Ag tolerance genes *silA-silE*±*pcoA-pcoD* were detected in 37% (n=101/275) of *Salmonella* isolates from diverse sources, serotypes (n=9/49; 18%) and clones (18 PFGE-types and 10 sequence types-ST), including recently emerging ones. Most of them were identified as *S*. Typhimurium "European clone" (ST34) and *S*. Rissen (ST469) (n=80/80-100% *versus* n=21/195-11% from other serotypes/clones). They were often recovered from samples of pig origin and were MDR (n=78/101; 77%) (Table 1). The simultaneous presence of *sil* and *pco* genes was frequently found (95%; n=96/101) and always associated with the complete *sil*+*pco* gene clusters. Combinations of *sil*+*pco*+*merA*±*terF* (40%; n=40/101) were frequently detected among diverse MDR serotypes/clones (6 serotypes/6 STs; 12 PFGE-types). In contrast, single carriage of *sil* (n=4/101) or *sil*+*merA* (n=1/101) genes was detected in only 5% of the

isolates, corresponding to the *S*. Typhimurium DT104 epidemic clone (ST19) with the typical ACSSuT (A-amoxicillin, C-chloramphenicol, S-streptomycin, Su-sulfamethoxazole and T-tetracycline) antibiotic resistance profile (related with SGI-1) apart from trimethoprim resistance (Table 1).

Salmonella (63%, n=174/275) without *sil*±*pco* belonged to 46 serotypes, including all the isolates from several poultry/eggs-associated serotypes such as *S*. Enteritidis, *S*. Hadar, *S*. Virchow, *S*. Infantis, *S*. Saintpaul, *S*. Stanley or *S*. Heidelberg. The analysis of antibiotic resistance profiles revealed that these isolates without *sil*±*pco* were frequently less MDR (n=84/174; 48%) than *sil*±*pco*$^+$ (Table S1, available as Supplementary data at JAC Online). Nonetheless, other acquired metal tolerance genes such as *merA* (26%; n=45/174) and/or *arsB*+*terF* (1%; n=1/174) were found in 26% (n=46/174) of these isolates, which belonged to 10 MDR serotypes (Table S1, available as Supplementary data at JAC Online).

Higher tolerance to *$CuSO_4$ in anaerobic atmosphere and to $AgNO_3$ after prior exposure to silver was observed among the sil+pco*$^+$ *or sil*$^+$ *isolates*

All *sil*+*pco*$^+$ or *sil*$^+$ isolates presented higher MIC_{CuSO4} values comparing with those without these genes (MIC_{50}/MIC_{90}=28/32 mM *versus* 2/8 mM, respectively) in anaerobic condition tests. The $MICs_{CuSO4}$ in aerobiosis were higher (MIC_{50}/MIC_{90}=32/36 mM) than in anaerobiosis, with identical MICs for isolates with or without *sil*±*pco* genes (Table 2). Genotypic assays also confirmed the occurrence of complete *sil*+*pco* gene clusters in isolates with MIC_{CuSO4}>16mM and its absence when the MICs were lower under anaerobiosis. The only exceptions were 2 isolates (*S*. Essen and *S*. II 4,5:b:-) lacking the known *sil*+*pco* gene clusters, but exhibiting high MIC_{CuSO4} values (24 and 28 mM) in anaerobic atmosphere (Table 1).

Regarding $AgNO_3$, differences between *sil*$^+$ (MIC_{50}/MIC_{90}=0.25 mM in aerobiosis and 0.32/0.5 mM in anaerobiosis) and *sil*$^-$ (MIC_{50}/MIC_{90}=0.125/0.16 mM in aerobiosis and 0.16/0.32 mM in anaerobiosis) isolates were observed in both atmospheres, although a significant increment was detected for *sil*$^+$ isolates only after prior contact with Ag (MIC_{50}/MIC_{90}>3mM in *sil*$^+$ isolates *versus* 0.125/0.16 mM in *sil*$^-$ isolates). One exception was one *Salmonella* isolate (*S*. Give) with previous contact with $AgNO_3$ and with a MIC that only increased up to 0.5 mM (Table 3).

Metal tolerance genes were located in diverse MDR genetic platforms

Among the characterised isolates, selected accordingly to the serotype and/or PFGE-type frequency, diverse genetic contexts for *sil*±*pco* genes were detected (Table 1).

Isolates of *S*. Typhimurium ("European clone"), *S*. Rissen, *S*. Anatum, *S*. Brandenburg, *S*. Bredeney, *S*. Give and S. Senftenberg, mostly resistant from 2 to 10 antibiotics, carried *sil*+*pco*±*merA*±*terF* genes in the chromosome. Additionally, these metal tolerance genes were often co-located in the same chromosomal I-*Ceu*I-PFGE fragment with diverse antibiotic resistant genes in the emergent *S*. Typhimurium "European clone" [ASSuT markers-bla_{TEM-1}, *strA*-*strB*, *tet*(B) and/or *sul2*] as well as in other serotypes [e.g. *tet*(A), *sul1*] (Table 1). In contrast, in isolates from 4 serotypes (*S*. Brandenburg, *S*. Bredeney, *S*. Muenchen and *S*. Wien) the *sil*+*pco*±*merA*±*terF* genes were plasmid located (190-270kb; IncHI1-n=1, IncHI2-n=2 or untypeable-n=1) along with variable combinations of typical and/or atypical *sul3*-type class 1 integrons plus other antibiotic resistance genes [e.g. bla_{TEM-1}, *tet*(A), *tet*(B)]. In *S*. Typhimurium DT104 MDR clone carrying only *sil*±*merA* genes they were found co-located in IncFIIA (n=1) or IncN (n=3) plasmids (100-140 kb) with typical and/or atypical *sul3*-type class 1 integrons[23] plus other antibiotic resistance genes [e.g. bla_{TEM-1}, *tet*(A)].

Isolates of diverse clinically relevant MDR *Salmonella* serotypes without *sil*±*pco* carried other metal tolerance genes (*merA* and/or *arsB*+*terF*). They were co-located with diverse antibiotic resistance genes (several associated with class 1 integrons) either in the chromosome or in plasmids of diverse families (80-290 kb; IncFIIs-n=5, IncI1-n=1, IncHI2-n=1, IncP-n=4, untypable-n=2) (Table S1, available as Supplementary data at JAC Online).

Copper and silver tolerance was co-transferred along with antibiotic resistance

The conjugative transfer of plasmid located *sil*+*pco* (n=3/4-IncHI1, IncHI2 or untypeable; one IncHI2 not transferred) and *sil* (n=1/4-IncFIIA; three IncN not transferred) genes was achieved for 50% of the *Salmonella* strains studied (n=4/8; *S*. Typhimurium, *S*. Bredeney, *S*. Muenchen and *S*. Wien), using both *E*. *coli* K802N and *S*. Typhimurium LSP 448/02 recipient strains (Table 4). Acquisition of *sil*±*pco* genes conferred higher MIC_{CuSO4} (20-28 mM) to transconjugants than the recipient strains *E*. *coli* K802N (1-2 mM) and *S*. Typhimurium LSP 448/02 (8-12 mM) in anaerobic atmosphere. Regarding MIC_{AgNO3}, *S*. Typhimurium LSP 448/02 transconjugants also presented a high MIC_{AgNO3} (>3 mM) after previous contact with Ag. Both *sil*+*pco* and antibiotic resistance genes (*sul*, *dfrA*, *aadA*, *tet*) were co-located in the same plasmid belonging to diverse families (IncFIIA, IncHI1, IncHI2 or untypeable plasmids) as in the donor strains (Table 4). None of the *sil*+*pco* genes located in the chromosome (n=5 tested) were transferable by conjugation assays, as well as an unknown Cu tolerance

mechanism observed in 2 *Salmonella* isolates (*S*. Essen and *S*. II 4,5:b:-) presenting high MIC_{CuSO4} in anaerobic conditions.

DISCUSSION

Using a large and extensively characterised *Salmonella* collection we address the potential role of metal tolerance, namely to Cu and Ag, for the emergence of particular *Salmonella* serotypes/clones associated with human infections and spread through the food chain. We also discussed the potential impact of this tolerance in the survival of such serotypes/clones in specific environments (e.g. contaminated with Cu under anaerobic conditions) as well as the diversity of genetic platforms implicated in the spread of metal tolerance genes along with antibiotic resistance among *Salmonella*.

In this study, we demonstrate that the Cu/Ag tolerance genes *sil±pco* were dispersed among diverse *Salmonella* serotypes and sources, mostly MDR, with emphasis to the clinically relevant emergent *S*. Rissen/ST469 and *S*. Typhimurium/ST34 "European clone". These two cases along with the emergent *S*. 4,[5],12:i:- MDR European and Spanish clones, previously described as often carrying *sil±pco* genes,[6] are strongly associated with pig production worldwide.[1,3,4,31] The higher European Cu concentrations allowed in feed for pigs (e.g. piglets 175 mg/kg, ~2.8 mM) than for other animals,[7] suggest the occurrence of a high selective pressure in the pig production setting. This selective pressure can potentially contribute to the emergence of such strains in pigs and piggeries related settings where Cu can rapidly accumulate, even at higher concentrations (e.g. up to 3387.6 mg/kg, 53 mM in pig manure and consequently pig amended soils).[14-15,32-34] In contrast, *S*. Enteritidis, the most reported serotype with poultry/eggs production as the main source[35], showed absence of *sil±pco* genes in our study and Genbank genome analysis (Table S3, available as Supplementary data at JAC Online). These data could be partial explained by the low Cu concentrations (e.g. recommendation for species or animal categories other than piglets is 35 mg/Kg, ~0.6 mM) allowed in this setting in Europe,[7] and/or be associated with particular features of this serotype impairing the acquisition and/or fixation of specific genetic platforms as those carrying *sil±pco*. However, the available genomes related to emerging poultry-related *Salmonella* serotypes in the USA (e.g. *S*. Heidelberg, *S*. Kentucky) carry *sil+pco* gene clusters (Table S3, available as Supplementary data at JAC Online).[36] This data alerts for the potential relevance of such genes in the survival and emergence of diverse *Salmonella* serotypes under different animal production practices (e.g. in the USA copper supplements are not restricted and the typical amounts range from 125-250 ppm, ~2-4 mM, in most broiler diets) or environmental factors.[37]

A common location of *sil*±*pco* on the chromosome was observed in diverse *Salmonella* serotypes from our collection, especially in the emergent MDR *S*. Rissen/ST469 and in the European clone of *S*. Typhimurium/ST34. This is in agreement with data previously reported for the major clone of *S*. 4,[5],12:i:- (European clone).[6] In other *Salmonella* serotypes/clones, *sil*±*pco* genes were located on large, mostly transferable, plasmids (100-270kb) of different major families (IncHI2, IncHI1, IncN, IncFIIA). These data point out to *sil*±*pco* widespread among diverse genetic platforms abundant in local metagenomes and described to be spread in *Enterobacteriaceae*.[38] In *Salmonella*, IncHI2 plasmids have been frequently associated with diverse metal tolerance genes as *sil*+*pco*,[36,39] but we highlight the relevance of IncFIIA and IncN plasmids in the dispersion of metal encoding genes, namely in the epidemic *S*. Typhimurium DT104 clone.

A more detailed genomic study (our isolates, Genbank genome analysis and literature available) showed that *sil* and *pco* gene clusters are often adjacent in *Salmonella* isolates from different sources and serotypes/clonal lineages, particularly in recently emerging ones (Table 1 and Table S3, available as Supplementary data at JAC Online).[6,19,40,41] The *in silico* analysis of recent available genome sequences pointed out, in most isolates, for a common module containing *sil*+*pco* gene clusters and genes coding for a hypothetical protein and a total/partial endonuclease (Table S3, available as Supplementary data at JAC Online). Such genes were described as part of a Tn*7*-like transposon previously identified in *Salmonella*, the Tn*6230*.[19,40,42] As showed in Table S3, available as Supplementary data at JAC Online, the complete Tn*6230* (common module with the typical Tn*6230* proteins TnsABC, involved in transposition of the genetic element) was present in most *sil*+*pco*$^{+}$ *Salmonella* isolates from diverse serotypes. Most of these Tn*6230* elements were near the *yhiN* gene, previously described to be the common insertion site of this transposon.[40,42] In contrast, when the TnsABC proteins were absent, the common module was usually located near a tRNA-Phe region or flanked by diverse insertion sequences (Table S3, available as Supplementary data at JAC Online). The absence of the TnsABC proteins could be related to recombination events, potentially associated with the fixation of the element in diverse *Salmonella* serotypes, namely in *S*. 4,[5],12:i:- and *S.* Typhimurium (Table S3, available as Supplementary data at JAC Online). These data, along with the chromosomal location of *sil*+*pco* genes in the emergent *S*. Rissen/ST469, *S*. Typhimurium/ST34 European clone and *S*. 4,[5],12:i:- European clone[6] from our collection, stresses the need of further studies to elucidate the contribution of these Tn*6230*-like elements alone, or eventually as part of potential genomic islands often related to Tn*7*-like transposons,[42] for the emergence of particular clones/serotypes.

Beside Cu and Ag tolerance genes (*sil*±*pco*) we frequently co-located other metal tolerance (e.g. *merA*) and antibiotic resistance genes (e.g. to tetracycline-*tetA*, *tetB;* sulphonamides-*sul1* and *sul3* class 1 integron related or *sul2*) in the same genetic platforms (plasmids or chromosome) of our isolates. In the case of the widespread European clone of *S*. Typhimurium, the chromosomal co-location of *sil*+*pco*, *merA* and ASSuT resistance region appears to be typical of these strains, as occurred with the European clone of *S*. 4,[5],12:i:-,[6] features that may have relevantly contributed to its evolution and emergence. Most of these antibiotic resistance and *merA* genes are described to be widespread among diverse bacterial hosts, genetic platforms and environments,[1,22,43,44] independently of *sil*+*pco* occurrence. This is also supported by our results showing several *Salmonella* serotypes carrying *sul*, *tet* and *merA* genes in diverse major plasmid families (IncP, IncFIIs, IncI1) without *sil*+*pco* genes. This abundance might facilitate their involvement in different recombination events with other metal encoding genetic elements (e.g. carrying Tn*6230*-like) and the maintenance of MDR isolates by the use of different compounds (e.g. antibiotics or metals). Recent environmental studies in metal contaminated soils/animal manure, particularly with Cu due to anthropogenic-derived sources (e.g. animal feed additives, agriculture microbicides), have shown that metal availability, even at low level concentrations, was correlated with a high prevalence of antibiotic resistance in diverse bacteria.[32-34,45,46] Also, an *in vitro* assay showed that the presence of sublethal levels of Cu or As were sufficient to co-select for the maintenance of MDR plasmids.[47]

Independently of plasmid and chromosomal location, the occurrence of *sil* efflux system was associated with a tolerance phenotype to $CuSO_4$ and $AgNO_3$ in wild type isolates (diverse *Salmonella* serotypes/clones, sources and antibiotic resistance profiles) and their transconjugants, pointing out to an acquired adaptive feature of these strains to diverse environmental challenges, beside antibiotic resistance. However, particular methodological conditions are mandatory to be applied in order to distinguish isolates with and without *sil* genes. In the case of $CuSO_4$, anaerobic conditions during phenotypic assays are critical, as under aerobiosis all isolates showed MIC≥20 mM (this study)[4,6,48] impairing the establishment of breakpoints values to distinguish isolates that have acquired Cu tolerance genes, namely *sil* or *sil*-*pco* gene clusters. Considering our results under anaerobiosis on an extensive collection of *Salmonella* serotypes (this study),[6] here we proposed a $CuSO_4$ tolerance cut-off>16 mM. However, as we did not identify any isolate with a MIC=16 mM, the inclusion of this value in the susceptible or tolerance group is not possible and we recommend the *sil* genes identification in future cases with MIC=16 mM. This approach under anaerobiosis was also critical in our previous study to differentiate *Enterococcus* spp.

with/without the Cu tolerance *tcrYAZB* cluster, which was not accurate under aerobic conditions.[49] In the case of $AgNO_3$ susceptibility assays, for the detection of a clear tolerance phenotype associated with the occurrence of *sil* efflux system, a prior contact of the isolates with subinhibitory concentrations of $AgNO_3$ is critical, as previously reported for *S*. 4,[5],12:i:-.[6] Using this approach, an $AgNO_3$ tolerance cut-off value >3 mM is proposed.

The increase tolerance of *Salmonella* isolates to $CuSO_4$ and $AgNO_3$ implies a better awareness of the potential role of these metal contaminants[13-15] for an improved survival, selection and competition of *sil*+ MDR emergent serotypes/clones than *sil*- ones in diverse environments (e.g. across the food chain), especially when under specific conditions.[4,8,9] For example, *Salmonella* isolates carrying *sil* cluster might overcome the highly toxic cuprous form Cu^+ abundant in anaerobic environments (e.g. gut of livestock carriers, manure, waste lagoons or forage/feed, sewage and sludge)[19,20,50] or silver salts/nanoparticles in diverse food production contexts (e.g. disinfectants used in water or surfaces in the animal production setting/environment; human and animal antiseptics).[8,51,52] However, the relevance of *pco* gene cluster for Cu tolerance in *Salmonella* under aerobiosis was not here demonstrated, corroborating other studies.[4,6] Additional research is needed to clarify the potential role of *pco* among *Salmonella* namely its involvement in virulence, as described for other multicopper oxidases.[53]

In summary, we report a high occurrence and co-location of copper/silver tolerance and antibiotic resistance genes among *Salmonella* from diverse serotypes, and their particular dispersion in the emergent MDR *S*. Rissen and *S*. Typhimurium clones commonly associated with pig production. The fact that recently emerging MDR serotypes are enriched in *sil*+*pco* gene clusters also alerts for the relevance of the dispersion and fixation of these genes in other less frequent serotypes and their potential emergence in a near future. The extensive copper use as feed additive and the low O_2 levels (e.g. animal gut/manure/waste lagoons) found in certain locations of food-animal producing environments might have contributed for the maintenance and dispersion of *sil*±*pco* carrying MDR *Salmonella* and/or Tn*6230*-like elements along with antibiotic resistance in the animal production setting, surrounding environments and food chain. We urgently need to be aware of the role of reducing environments under multiple stressors (e.g. occurrence of the more toxic Cu^+ or antibiotics) for an enhanced survival, selection and persistence of particular copper tolerant-MDR strains. An assessment of control measures for the use and/or accumulation of metals in diverse environments to prevent a wider expansion of MDR *S*. Rissen and *S*. Typhimurium clones or the emergence of new ones is also required.

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TRANSPARENCY DECLARATIONS

None to declare.

SUPPLEMENTARY DATA

Tables S1, S2 and S3 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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Table 1. Epidemiological features and genotypic/phenotypic characterization of *Salmonella enterica* isolates carrying acquired copper and silver tolerance genes (*sil* and *pco*)

Serotype (no. isolates)	**"Clone designation" and PFGE-type** (no. isolates)**/ST**[a]	**Isolation date; region**[b]	**Source/sample** (no. isolates)[c]	**Antibiotic resistance profile/genes** (no. isolates)[d]	**Cu/Ag and other metal tolerance genes** (no. isolates)	**MIC_{range} $CuSO_4$ Aerobic/anaerobic (mM)** (no. isolates)	**MIC_{range} $AgNO_3$ Aerobic/with Ag contact (mM)** (no. isolates)	**Location of metal tolerance genes + co-location with antibiotic resistance genes – Chr or PL (Kb, Inc)** (no. isolates)[e]
Typhimurium (n=37)	"European clone"- A (n=8), B (n=11), C (n=8), G (n=2) and F (n=1)/ST34	2003-2004, 2007-2013; N, C, S, I	Animal production/Pi (n=16); Food/B and U (n=2); Human/ C (n=1) and H (n=11)	AMX, STR, SUL, TET, [GEN, KAN, NAL, CIP^{DS}]/bla_{TEM-1}, *strA-strB, sul2, tet*(B) [*tet*(A), *aac(3)-IV*, *aphA1*] (n=21) AMX, STR, SUL/bla_{TEM-1}, *strA-strB, sul2* (n=1) AMX, SUL, TET/bla_{TEM-1}, *sul2, tetB* (n=6) SUL, TET/*tet*(B) (n=1) Susceptible (n=1)	*pcoA-pcoD* + *silA-silE* + *merA* (n=27) *pcoA-pcoD* + *silA-silE* (n=3)	28-32/20-32 (n=11)	0.125-0.5 (n=11)/>3 (n=4)	*pcoD*, *silA*, *merA* + bla_{TEM-1}, *strA-strB*, *sul2*, *tet*(B) – Chr (n=3)
	Other - TB (n=2)/ST34	2012; S	Human/H (n=2)	AMX, CHL, CIP, GEN, KAN, STR, SUL, TET, TMP, NAL/bla_{TEM-1}-bla_{OXA-30}, *oqxAB*, *aac(6')-Ib-cr*, *catB3*, *cmlA1*, *floR*, *aac(3)-IV*, *aphA1*, *aadA*, *strA-strB*, *sul1-sul2-sul3*, *tet*(B), *dfrA12* (n=2)	*pcoA-pcoD* + *silA-silE* + *merA* + *terF* (n=2)	36/24 (n=2)	0.125 (n=2)/>3 (n=1)	*pcoD*, *silA*, *merA* + bla_{TEM-1}, *strA-strB, tet*(B) – Chr; *terF* + bla_{OXA-30}, *oqxAB, aac(6')-Ib-cr, catB3, cmlA1, floR, aac(3)-IV, aphA1, aadA, sul1-sul2-sul3, dfrA12* – PL (180, HI2) (n=2)
	"DT104" – A (n=5)/ST19	2003-2004; N	Food/P and U (n=2); Human/H (n=3)	AMX, CHL, STR, SUL, TET, TMP/bla_{TEM-1}-bla_{PSE-1}, *floR*, *aadA2*, *sul1-sul3*, *tet*(G), *dfrA12* (n=1) AMX, CHL, STR, SUL, TET, TMP/bla_{TEM-1}, *cmlA1*, *aadA2*-[*aadA1*], *sul1*-[*sul3*], *tet*(A), *dfrA12* (n=4)	*silA-silE* + *merA* (n=1) *silA-silE* (n=4)	28-36/24-32 (n=5)	0.125-0.25 (n=5)/>3 (n=2)	*silA, merA* + bla_{TEM-1}, *sul3, dfrA12* – PL (100, FIIA) (n=1) *silA* + bla_{TEM-1}, *cmlA1*, [*aadA1-aadA2*], [*sul1-sul3*], *tet*(A), *dfrA12*[f] – PL (140, N) (n=3)
Rissen (n=50)	N (n=49)/ST469	2002-2004, 2006, 2007-2013; N, C, S	Animal production/Pi (n=9); Food/ Cw, CM, P, Pt and U (n=25); Environment/W (n=1) and WR (n=1); Human/C (n=3) and H (n=10)	AMX, STR, SUL, TET, TMP, [CHL]/bla_{TEM-1}, *aadA2*, *sul1*, *tet*(A), *dfrA12*, [*aadA1*, *sul3*, *catA-cmlA1*] (n=24) AMX, STR, SUL, TET, TMP/ bla_{TEM-1}, *aadA5*, *sul1*, *tet*(A), *dfrA17* (n=1) CHL, STR, SUL, TET, TMP/*cmlA1*, *aadA2-aadA1*, *sul3*, *tet*(A), *dfrA12* (n=2) AMX, SUL, TET, TMP/bla_{TEM-1}, *sul1*, *tet*(A), *dfrA12*, *aadA2* (n=1)	*pcoA-pcoD* + *silA-silE* + *merA* (n=2) *pcoA-pcoD* + *silA-silE* (n=47)	24-36/24-36 (n=33)	0.25-0.32 (n=33)/>3 (n=6)	*pcoD*, *silA* + [*tet*(A)] – Chr (n=14)

Table 1. *Continued*

Serotype (no. isolates)	**"Clone designation" and PFGE-type** (no. isolates)/**ST**[a]	**Isolation date; region**[b]	**Source/sample** (no. isolates)[c]	**Antibiotic resistance profile/genes** (no. isolates)[d]	**Cu/Ag and other metal tolerance genes** (no. isolates)	**MIC_{range} $CuSO_4$ Aerobic/anaerobic (mM)** (no. isolates)	**MIC_{range} $AgNO_3$ Aerobic/with Ag contact (mM)** (no. isolates)	**Location of metal tolerance genes + co-location with antibiotic resistance genes – Chr or PL (Kb, Inc)** (no. isolates)[e]
				AMX, STR, TET, TMP/*bla*$_{TEM-1}$, *aadA2*, *tet*(A), *dfrA12* (n=1) AMX, TET, CHL, STR/ *bla*$_{TEM-1}$, *tet*(A) (n=1) SUL, TET, TMP, [STR]/*sul1*, *tet*(A), *dfrA12*, *aadA2* (n=4) AMX, SUL, TMP/*bla*$_{TEM-1}$, *sul1*, *dfrA12, aadA2* (n=1) TET, [STR]/*tet*(A), [*aadA2*] (n=11) Susceptible (n=3)				
	Z (n=1)/ST469	2013; S	Human/H (n=1)	AMX, STR, SUL, TET, TMP/*bla*$_{TEM-1}$, *aadA2*, *sul1*, *tet*(A), *dfrA12* (n=1)	*pcoA-pcoD* + *silA-silE* (n=1)	36/32 (n=1)	0.25/>3 (n=1)	*pcoD*, *silA* + *bla*$_{TEM-1}$ - Chr (n=1)
Anatum (n=2)	M (n=1)/ST64	2004; N	Human/C (n=1)	AMX, CHL, STR, SUL, TET, TMP/*bla*$_{TEM-1}$, *aadA5*, *sul1*, *tet*(A), *dfrA17* (n=1)	*pcoA-pcoD* + *silA-silE* + *merA* (n=2)	28-36/24-32 (n=2)	0.25-0.5/>3 (n=2)	*pcoD*, *silA*, *merA* + *aadA5*, *sul1*, *dfrA17*[f] - Chr (n=1)
	Y (n=1)/ST64	2002; N	Food/P (n=1)	SUL, TET, TMP/*sul1*, *tet*(A), *dfrA17* (n=1)				*pcoD*, *silA*, *merA* + *sul1*, *tet*(A) – Chr (n=1)
Brandenburg (n=2)	G (n=1)/ST334	2003; N	Food/P (n=1)	AMX, CHL, STR, SUL, TET, TMP/*bla*$_{TEM-1}$, *aadA1*, *sul1*, *tet*(A), *dfrA1* (n=1)	*pcoA-pcoD* + *silA-silE* + *merA* + *terF* (n=1)	32/24 (n=2)	0.16/>3 (n=2)	*pcoD*, *silA*, *merA*, *terF* + *bla*$_{TEM-1}$, *aadA1*, *sul1*, *dfrA1*[f] – PL (260, HI2) (n=1)
	V (n=1)/ST334	2004; N	Human/H (n=1)	SUL, TET, TMP/*sul1*, *tet*(A), *dfrA1*, *aadA1* (n=1)	*pcoA-pcoD* + *silA-silE* + *merA* (n=1)			*pcoD*, *silA*, *merA* + *sul1*, *dfrA1*, *aadA1*[f] – Chr (n=1)
Bredeney (n=4)	L (n=4)/ST306	2002, 2004, 2010; N	Food/OT (n=1); Human/H (n=3)	SUL, TET, TMP/*sul1*, *tet*(A), *dfrA17*, *aadA5* (n=1) TET, TMP/*tet*(A), *dfrA17*, *sul1*, *aadA5* (n=2) SUL, TMP/*sul1*, *dfrA17* (n=1)	*pcoA-pcoD* + *silA-silE* + *merA* + *terF* (n=2) *pcoA-pcoD* + *silA-silE* + *merA* (n=2)	28-36/28-32 (n=4)	0.125-0.25 (n=4)/>3 (n=1)	<u>*pcoD, silA, merA + sul1, tet*(A), *dfrA17, aadA5*[f] - PL (195, UN)</u>; *terF* - Chr (n=1) *pcoD*, *silA*, *merA*, [*terF*] + *sul1*, *dfrA17*, [*aadA5*][f] – Chr (n=3)
Give (n=1)	U (n=1)/STNew	2010; C	Food/U (n=1)	Susceptible (n=1)	*pcoA-pcoD* + *silA-silE* (n=1)	36/32 (n=1)	0.25/0.5 (n=1)	*pcoD*, *silA* - Chr (n=1)
Muenchen (n=1)	D (n=1)/ST82	2003; N	Human/H (n=1)	CHL, STR, SUL, TMP/*catA, aadA1*, *sul1, dfrA1* (n=1)	*pcoA-pcoD* + *silA-silE* + *merA* + *terF* (n=1)	36/32 (n=1)	0.32/>3 (n=1)	<u>*pcoD, silA, merA, terF + catA, aadA1, sul1, dfrA1*[f] – PL (270, HI2)</u> (n=1)
Senftenberg (n=3)	SA (n=3)/ST14	2002-2003; N, C	Animal production/Pi (n=1); Food/Pt (n=2)	NAL, CIP^{DS}/ND (n=1) Susceptible (n=2)	*pcoA-pcoD* + *silA-silE* (n=3)	32-36/32 (n=3)	0.25-0.32 (n=3)/>3 (n=1)	*pcoD*, *silA* - Chr (n=3)

Table 1. *Continued*

Serotype (no. isolates)	**"Clone designation" and PFGE-type** (no. isolates)/**ST**[a]	**Isolation date; region**[b]	**Source/sample** (no. isolates)[c]	**Antibiotic resistance profile/genes** (no. isolates)[d]	**Cu/Ag and other metal tolerance genes** (no. isolates)	**MIC_{range} $CuSO_4$ Aerobic/anaerobic (mM)** (no. isolates)	**MIC_{range} $AgNO_3$ Aerobic/with Ag contact (mM)** (no. isolates)	**Location of metal tolerance genes + co-location with antibiotic resistance genes – Chr or PL (Kb, Inc)** (no. isolates)[e]
Wien (n=1)	J (n=1)/ST102	2003; S	Food/P (n=1)	AMX, CHL, KAN, STR, SUL, TET, TMP/bla_{TEM-1}, *cmlA1-catA*, *aphA1*, *aadA2-aadA1*, *sul1-sul3*, *tet*(B), *dfrA1* (n=1)	*pcoA-pcoD* + *silA-silE* (n=1)	32/28 (n=1)	0.25/>3 (n=1)	*pcoD*, *silA* + bla_{TEM-1}, *cmlA1-catA*, *aphA1*, *aadA2-aadA1*, *sul1-sul3*, *tet*(B), *dfrA1*[f] – PL (250, HI1) (n=1)

AMX, amoxicillin; CHL, chloramphenicol; CIP, ciprofloxacin; CIP^{DS}, decreased susceptibility to ciprofloxacin, GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TMP, trimethoprim; Chr, chromosome; PL, plasmid; ND, not determined; UN, untypable.

[a]"Clone designation" was adopted only when has been previously published. PFGE-types were designated by capital letters, including previously described[1,22,23] or firstly designated in this study.

[b]N, north; C, centre; S, south; I, islands (Azores).

[c]B, beef; C, community; CM, cooked meal; Cw, cow; H, hospitals patients; OT, other type of food; P, pork; Pi, pigs and piggeries environment; Pt, poultry; U, unknown; W, bathing or drinking water; WR, river water.

[d]Variable antibiotic resistance phenotypes and genotypes were presented between square brackets. Isolates resistant to three or more antibiotics from different families were considered multidrug-resistant.

[e]Variable metal tolerance genes were presented between square brackets. Underlined metal tolerance or antibiotic resistance genes were also detected in transconjugants.

[f]In particular serotypes/clonal lineages *silA*±*pcoD*, *merA* or *terF* were in the same genetic platform that the genes belonging to conventional class 1 integrons [1000bp (*aadA1*) in *S*. Muenchen; 1000bp (*aadA2*) in *S*. Typhimurium "DT104" clone; 1700bp (*dfrA1-aadA1*) in *S*. Brandenburg and *S*. Wien and 1800bp (*dfrA17-aadA5*) in *S*. Bredeney and *S*. Anatum] or to atypical *sul3* [type I integron 7085bp (*dfrA12-orfF-aadA2-cmlA1-aadA1-qacH*-IS440-*sul3*) in *S*. Typhimurium "DT104" clone and type III integron 7304bp (*estX-psp-aadA2-cmlA1-aadA1-qacH*-IS440-*sul3*)] in *S*. Wien].[22,23]

Table 2. Minimum inhibitory concentrations of $CuSO_4$ for *Salmonella enterica* isolates

Serotype	Environment	*silA-silE*	*pcoA-pcoE*	No. of Isolates[a]	MIC (mM)[b]												MIC_{50} (mM)	MIC_{90} (mM)
					0.5	1	2	4	8	12	16	20	24	28	32	36		
Typhimurium	Aerobic	+	+	13										4	7	2	32	36
		+	-	5										2	2	1	32	36
		-	-	32								1		10	20	1	32	32
	Anaerobic	+	+	13								1	10	1	1		24	28
		+	-	5									1	3	1		28	32
		-	-	32			1	18	13								4	8
Rissen	Aerobic	+	+	34									1		21	12	32	36
	Anaerobic	+	+	34									1	19	13	1	28	32
Enteritidis	Aerobic	-	-	14										10	4		28	32
	Anaerobic	-	-	14		4	8	2									2	4
Other	Aerobic	+	+	14										2	5	7	32	36
		-	-	54										5	26	23	32	36
	Anaerobic	+	+	14									3	4	7		28	32
		-	-	54		13	33	2	3	1			1[c]	1[c]			2	8
TOTAL	Aerobic	+	+ or -	66									1	8	35	22	32	36
		-	-	100								1		25	50	24	32	36
	Anaerobic	+	+ or -	66								1	15	27	22	1	28	32
		-	-	100		17	42	22	16	1			1[c]	1[c]			2	8

[a]The number of isolates included representatives of all the serotypes: *Salmonella enterica* Typhimurium (n=50/96), *Salmonella enterica* Rissen (n=34/50), *Salmonella enterica* Enteritidis (n=14/32) and other *Salmonella enterica* serotypes (n=68/97).

[b]*Escherichia coli* ED8739 (plasmid pRJ1004 with *pco* genes; MIC_{CuSO4} = 32-36 mM, aerobiosis)[28] and *Enterococcus faecium* BM4105RF (negative for all genes tested; MIC_{CuSO4} = 8-12 mM, aerobiosis)[26] were used as control strains in Cu assays; Those strains were also used in anaerobic assays: *E. coli* ED8739 (plasmid pRJ1004 with *pco* genes; MIC_{CuSO4} = 16-20 mM) and *E. faecium* BM4105RF (MIC_{CuSO4} = 2-4 mM).[6] A PCR approach confirmed the presence or absence of *silCPRS* and *pcoRSE* gene clusters in representative isolates of all anaerobic MIC.

Table 2. *Continued*

The corresponding CuSO4 mg/L values for the mM-values tested are: 0.25 mM-39.90 mg/L, 0.5 mM-79.80 mg/L, 1 mM-159.61 mg/L, 2 mM-319.22 mg/L, 4 mM-638.44 mg/L, 8 mM-1276.87 mg/L, 12 mM-1915.308 mg/L, 16 mM-2553.74 mg/L, 20 mM-3192.18 mg/L, 24 mM-3830.62 mg/L, 28 mM-4469.05 mg/L, 32 mM-5107.49 mg/L, 36 mM-5745.92 mg/L.

[c]Isolates without amplification of *silCPRS* and *pcoRSE* gene clusters by PCR.

Table 3. Minimum inhibitory concentrations of $AgNO_3$ for *Salmonella enterica* isolates

Serotype	Environment	*silA-silE*	No. of Isolates[a]	MIC (mM)[b]										MIC_{50} (mM)	MIC_{90} (mM)
				0.08	0.125	0.16	0.25	0.32	0.5	0.75	1	1.5	>3		
Typhimurium	Aerobic	+	18		5	2	9		2					0.25	0.5
		-	32		13	19								0.16	0.16
	Anaerobic	+	18		2	1	2		13					0.5	0.5
		-	32		11	2	18		1					0.25	0.25
	Aerobic/previous contact	+	7										7	>3	>3
	with 0.025 mM $AgNO_3$	-	4		4									0.125	0.125
Rissen	Aerobic	+	34				33	1						0.25	0.25
	Anaerobic	+	34				11	11	11	1				0.32	0.5
	Aerobic/previous contact with 0.025 mM $AgNO_3$	+	7										7	>3	>3
Enteritidis	Aerobic	-	14		12	2								0.125	0.16
	Anaerobic	-	14			1	13							0.25	0.25
	Aerobic/previous contact with 0.025 mM $AgNO_3$	-	6		5	1								0.125	0.16
Other	Aerobic	+	14		1	2	8	2	1					0.25	0.32
		-	54		33	16	5							0.125	0.16
	Anaerobic	+	14			3	3		7	1				0.5	0.5
		-	54	24	5	10	2	8	5					0.125	0.32
	Aerobic/previous contact	+	9						1				8	>3	>3
	with 0.025 mM $AgNO_3$	-	5		3	2								0.125	0.16
TOTAL	Aerobic	+	66		6	4	50	3	3					0.25	0.25
		-	100		58	37	5							0.125	0.16
	Anaerobic	+	66		2	4	16	11	31	2				0.32	0.5
		-	100	24	16	13	33	8	6					0.16	0.32
	Aerobic/previous contact	+	23						1				22	>3	>3
	with 0.025 mM $AgNO_3$	-	15		12	3								0.125	0.16

Table 3. *Continued*

[a]The number of isolates included representatives of all the serotypes: *Salmonella enterica* Typhimurium (n=50/96), *Salmonella enterica* Rissen (n=34/50), *Salmonella enterica* Enteritidis (n=14/32) and other *Salmonella enterica* serotypes (n=68/97).

[b]*Escherichia coli* J53 (plasmid pMG101 with *sil* genes; MIC_{AgNO3}>3 mM, aerobiosis)[27] and *Staphylococcus aureus* ATCC 29213 [negative for all genes tested; MIC_{AgNO3}=0.16-0.25 mM, aerobiosis][27] were used as control strains in Ag assays. Those strains were also used in anaerobic assays: *E. coli* J53 (plasmid pMG101 with *sil* genes; MIC_{AgNO3}>3 mM) and *S. aureus* ATCC 29213 (MIC_{AgNO3}=0.32-0.5 mM).[6] A PCR approach confirmed the presence or absence of *silCPRS* gene cluster in representative isolates of all MIC after previous contact with Ag. The corresponding mg/L values for the mM-values tested are: 0.08 mM-13.59 mg/L, 0.125 mM-21.23 mg/L, 0.16 mM-27.18 mg/L, 0.25 mm-42.47 mg/L, 0.32 mM-54.36 mg/L, 0.5 mM-84.94 mg/L, 0.75 mM-127.40 mg/L, 1 mM-169.87 mg/L, 1.5 mM-254.81 mg/L, >3 mM-509.62 mg/L.

Table 4. Co-transfer of copper and silver tolerance with antibiotic resistance

***Salmonella* serotypes** (no. of positive mating assays)	**Compounds used as selective agents**	**Recipient strains used**[a]	**TRANSCONJUGANTS**						
			MIC_{CuSO4} (mM)		**MIC_{AgNO3} (mM)**				
			Aerobiosis	**Anaerobiosis**	**Aerobiosis**	**Aerobiosis with Ag contact**	**Cu/Ag and other metal tolerance genes**	**Antibiotic resistance phenotype**	**Location of metal tolerance genes + co-location with antibiotic resistance genes – PL (Kb, Inc)**
Typhimurium (1/3)	SUL (256 mg/L)	*E. coli* K802N	24	20	0.125	[b]	*silA*+*merA*	AMX, SUL, TMP, NAL	*silA*, *merA* + bla_{TEM}, *sul3*, *dfrA12* – PL (100, FIIA)
	TMP (8 mg/L)	*S. enterica* LSP 448/02	>36	28	0.25	>3	*silA*+*merA*	AMX, SUL, TMP, NAL	
Bredeney (1/1)	SUL (256 mg/L)	*E. coli* K802N	24	20	0.08	[b]	*pcoD*+*silA*+*merA*	SUL, TET, TMP, NAL	*pcoD*, *silA*, *merA* + *sul1*, *tet*(A), *dfrA17*, *aadA5*[c] – PL (195, UN)
	TMP (8 mg/L)	*S. enterica* LSP 448/02	>36	28	0.25	>3	*pcoD*+*silA*+*merA*	SUL, TET, TMP, NAL	
Muenchen (1/1)	SUL (256 mg/L)	*E. coli* K802N	24	20	0.08	[b]	*pcoD*+*silA*+*merA*+*terF*	CHL, STR, SUL, TMP, NAL	*pcoD*, *silA*, *merA*, *terF* + *catA*, *aadA1*, *sul1*, *dfrA1*[c] – PL (270, HI2)
	STR (32 mg/L)	*S. enterica* LSP 448/02	>36	28	0.25	>3	*pcoD*+*silA*+*merA*+*terF*	CHL, STR, SUL, TMP, NAL	
	TMP (8 mg/L)	*S. enterica* LSP 448/02	>36	28	0.25	>3	*pcoD*+*silA*+*merA*+*terF*	CHL, STR, SUL, TMP, NAL	
Wien (1/1)	SUL (256 mg/L)	*E. coli* K802N	24	20	0.08	[b]	*pcoD*+*silA*	AMX, CHL, KAN, STR, SUL, TET, TMP, NAL	*pcoD*, *silA* + bla_{TEM}, *cmlA1-catA*, *aphA1*, *aadA2-aadA1*, *sul1-sul3*, *tet*(B), *dfrA1*[c] – PL (250, HI1)
	AMX (16 mg/L)	*S. enterica* LSP 448/02	>36	28	0.25	>3	*pcoD*+*silA*	AMX, CHL, KAN, STR, SUL, TET, TMP, NAL	

AMX, amoxicillin; CHL, chloramphenicol; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TMP, trimethoprim; PL, plasmid; UN, untypeable.

Table 4. *Continued*

[a]Antibiotic resistance and metal tolerance of the recipient strains used in conjugation assays: *Escherichia coli* K802N (rifampicin and nalidixic acid resistant; MIC_{CuSO4}=24-28 mM, aerobiosis and MIC_{CuSO4}=1-2 mM, anaerobiosis; MIC_{AgNO3}=0.08-0.125 mM, aerobiosis); *Salmonella* Typhimurium LSP 448/02 (nalidixic acid resistant; MIC_{CuSO4}=32-36 mM, aerobiosis and MIC_{CuSO4}=8-12 mM, anaerobiosis; MIC_{AgNO3}=0.16-0.25 mM, aerobiosis and $MIC_{Ag\ contact}$=0.125-0.25 mM).

[b]The described Ag protocol assay was designed for testing *Salmonella* isolates.

[c]In particular serotypes/clonal lineages *silA*±*pcoD*, *merA* or *terF* were in the same genetic platform that the genes belonging to conventional class 1 integrons [1000bp (*aadA1*) in *S*. Muenchen; 1700bp (*dfrA1-aadA1*) in *S*. Wien and 1800bp (*dfrA17-aadA5*) in *S*. Bredeney] or to atypical *sul3* [type III integron 7304bp (*estX-psp-aadA2-cmlA1-aadA1-qacH*-IS440-*sul3*)] in *S*. Wien.[22,23]

Table S1. Epidemiological features and genotypic/phenotypic characterization of *Salmonella enterica* isolates with absence of acquired copper and silver tolerance genes (*sil* and *pco*)

Serotype (no. isolates)	**"Clone designation" and PFGE-type** (no. isolates)**/ST**[a]	**Isolation date; region**[b]	**Source/sample** (no. isolates)[c]	**Antibiotic resistance profile/genes** (no. isolates)[d]	**Other metal tolerance genes + co-location with antibiotic resistance genes - Chr or PL (Kb, Inc)** (no. isolates)[e]	**MIC_{range} $CuSO_4$ Aerobic/anaerobic (mM)** (no. isolates)	**MIC_{range} $AgNO_3$ Aerobic/with Ag contact (mM)** (no. isolates)
Typhimurium (n=59)	"DT104"-A (n=28)/ST19	2002-2004, 2007-2014; N, C, S, I	Animal production/Pi (n=9); Food/P or U (n=4); Human/ C (n=2) and H (n=13)	AMX, CHL, STR, SUL, TET, [GEN, NAL, CIP^{DS}]/bla_{PSE-1}, *floR, aadA2, sul1*, *tet*(G), [*aac(3)-IV*, *sul2*] (n=22) AMX, CHL, STR, SUL/bla_{PSE-1}, *floR, aadA2, sul1* (n=1) AMX, STR, SUL, [NAL]/bla_{PSE-1}, *aadA2, sul1* (n=2) AMX, SUL/bla_{PSE-1}, *sul1* (n=1) STR, SUL/*aadA2, sul1* (n=2)	(-) (n=28)	28-36/4-8 (n=14)	0.125-0.16 (n=14)/0.125 (n=2)
	"DT104"-Q (n=1)/ST19	2000; N	Human/H (n=1)	CHL, KAN, SUL, TMP/*cmlA1, aphA1, sul3*, *dfrA12, aadA2-aadA1* (n=1)	*merA* + *cmlA1, aphA1, sul3, dfrA12, aadA2-aadA1*[f] – PL (100, FIIs) (n=1)	32/2 (n=1)	0.16/ND (n=1)
	"OXA-30-producing"-T (n=18)/ST19	2002-2004, 2008-2013; N, C, S	Food/P or Pt (n=4); Environment/U (n=1); Human/H (n=13)	AMX, CHL, STR, SUL, TET, [GEN]/bla_{OXA-30}, *catA, aadA1, sul1, tet*(B), [bla_{CMY-2}, *floR*, *sul2*, *aac(3)-IV*] (n=15) AMX, CHL, STR, SUL/bla_{OXA-30}, *catA, aadA1*, *sul1* (n=1) AMX, STR, SUL, TET/bla_{OXA-30}, *aadA1, sul1, tet*(B) (n=1) AMX, STR, SUL/ bla_{OXA-30}, *aadA1, sul1* (n=1)	*merA* + bla_{OXA-30}, *catA, aadA1, sul1, tet*(B)[f] – PL (100-140, FIIs) (n=4) *merA* – ND (n=14)	28-32/4-8 (n=9)	0.125-0.16 (n=9)/0.125 (n=1)

Serotype (no. isolates)	**"Clone designation" and PFGE-type** (no. isolates)**/ST**[a]	**Isolation date; region**[b]	**Source/sample** (no. isolates)[c]	**Antibiotic resistance profile/genes** (no. isolates)[d]	**Other metal tolerance genes + co-location with antibiotic resistance genes - Chr or PL (Kb, Inc)** (no. isolates)[e]	**MIC_{range} $CuSO_4$ Aerobic/anaerobic (mM)** (no. isolates)	**MIC_{range} $AgNO_3$ Aerobic/with Ag contact (mM)** (no. isolates)
	"Other" – Z (n=2)/ST19, AT/ND (n=1), AM/ND (n=1) and ND (n=8)	2002-2004, 2010, 2012, 2013; N, S	Food/Cw, P or Q (n=3); Environment/W (n=2); Human/H (n=7)	AMX, CHL, STR, SUL, TET, TMP/*bla*$_{TEM-1}$, *cmlA1, aadA2-aadA1*, *sul3*, *tet*(A), *dfrA12*, [*sul2*] (n=2) AMX, STR, SUL, TET/*bla*$_{TEM-1}$, *strA-strB*, *sul2, tet*(A) (n=2) AMX, SUL, TET, TMP/*bla*$_{TEM-1}$, *sul2, tet*(A) (n=2) AMX, TET/*bla*$_{TEM-1}$, *tet*(A) (n=1) TET/*tet*(A) (n=1) AMX/*bla*$_{TEM-1}$ (n=1) NAL, CIP^{DS}/ND (n=2) Susceptible (n=1)	(-) (n=12)	20-32/4-8 (n=8)	0.125-0.16 (n=8)/0.125 (n=1)
Enteritidis (n=32)	C (n=10)/ST11	2002-2004, 2008; N, S, Unknown	Animal production/Pi (n=1); Food/Pt or U (n=3); Human/H (n=6)	SUL, TET, TMP, [NAL, CIP^{DS}]/*sul1, tet*(A), *dfrA1*, *aadA1* (n=6) AMX, STR, SUL, TMP/*bla*$_{TEM-1}$, *aadA1*, *sul1-sul2, dfrA1* (n=1) AMX, STR, SUL, NAL, CIP^{DS}/*bla*$_{TEM-1}$, *aadA1*, *sul1* (n=1) NAL, TMP, CIP^{DS}/*qnrS1, dfrA14* (n=1) NAL, CIP^{DS}/ND (n=1)	*merA* + *sul1, tet*(A), *dfrA1, aadA1*[f] – PL (80, P) (n=1) *merA* – ND (n=6) (-) (n=3)	28-32/1-2 (n=4)	0.125-0.16 (n=4)/0.125 (n=3)
	K (n=1)/ST11	2003; C	Human/H (n=1)	SUL, TET/*sul1, tet*(A), *dfrA1, aadA1* (n=1)	*merA* + *sul1, tet*(A), *dfrA1, aadA1*[f] – PL (80, P) (n=1)	28/1 (n=1)	0.125/0.125 (n=1)
	ND (n=21)	2002-2003; N, S	Human/H (n=11) and C (n=2); Food/P, Pt or OT (n=8)	AMX, SUL, TET, TMP/*bla*$_{TEM-1}$, *sul2*, *tet*(A) (n=1) STR, SUL, TET, NAL, CIP^{DS}/*strA-strB*, *sul2, tet*(A) (n=1) AMX, GEN/*bla*$_{TEM-1}$, *aac(3)-IV* (n=1) AMX, [NAL, CIP^{DS}]/*bla*$_{TEM-1}$ (n=4) CHL, NAL, CIP^{DS}/(-) (n=1) TET, [NAL, CIP^{DS}]/*tet*(A) (n=2) NAL, CIP^{DS}/ND (n=6) Susceptible (n=5)	(-) (n=21)	28-32/2-4 (n=9)	0.125-0.16 (n=9)/0.125 (n=2)
Adelaide (n=1)	AA (n=1)/ST440	2012; N	Human/H (n=1)	Susceptible (n=1)	(-) (n=1)	28/2 (n=1)	0.25/0.125 (n=1)
Agona (n=1)	ND (n=1)	2010; S	Human/H (n=1)	Susceptible (n=1)	(-) (n=1)	32/2 (n=1)	0.125/ND (n=1)

Serotype (no. isolates)	**"Clone designation" and PFGE-type** (no. isolates)/**ST**[a]	**Isolation date; region**[b]	**Source/sample** (no. isolates)[c]	**Antibiotic resistance profile/genes** (no. isolates)[d]	**Other metal tolerance genes + co-location with antibiotic resistance genes - Chr or PL (Kb, Inc)** (no. isolates)[e]	**MIC_{range} $CuSO_4$ Aerobic/anaerobic (mM)** (no. isolates)	**MIC_{range} $AgNO_3$ Aerobic/with Ag contact (mM)** (no. isolates)
Anatum (n=1)	S (n=1)/ST64	2003; S	Human/H (n=1)	CHL, STR, SUL, TMP/*cmlA1, aadA2-aadA*1, *sul3*, *dfrA12* (n=1)	*merA* + *cmlA1, aadA2-aadA1, sul3, dfrA12*[f] – PL (120, I1) (n=1)	32/1 (n=1)	0.16/0.16 (n=1)
Bovismorbificans (n=1)	W (n=1)/ST142	2011; N	Human/H (n=1)	AMX, CTX, STR, SUL, GEN, KAN/$bla_{CTX-M-9}$, *aadB-aadA2, sul1* (n=1)	*arsB* + *terF* + $bla_{CTX-M-9}$, *aadB, aadA2, sul1*[f] – PL (240, HI2) (n=1)	32/8 (n=1)	0.16/0.125 (n=1)
Brandenburg (n=1)	ND (n=1)	2003; S	Food/P (n=1)	STR, SUL, TET/*strA-strB, sul2, tet*(A) (n=1)	(-) (n=1)	36/2 (n=1)	0.16/ND (n=1)
Bredeney (n=1)	ND (n=1)	2003; N	Food/P (n=1)	Susceptible (n=1)	(-) (n=1)	32/2 (n=1)	0.16/ND (n=1)
Brikama (n=1)	ND (n=1)	2011; S	Human/H (n=1)	Susceptible (n=1)	(-) (n=1)	36/2 (n=1)	0.125/ND (n=1)
Budapest (n=2)	BA (n=1)/ST1672	2011; N	Animal production/A (n=1)	STR/(-) (n=1)	(-) (n=1)	36/2 (n=1)	0.125/ND (n=1)
	ND (n=1)	2011; N	Human/H (n=1)	Susceptible (n=1)	(-) (n=1)	36/2 (n=1)	0.125/ND (n=1)
Derby (n=16)	B (n=14)/ST40	2003-2004, 2007-2008; N, S	Animal production/Pi (n=11); Food/P (n=2); Human/H (n=1)	AMX, STR, SUL, TET, GEN, KAN/bla_{TEM-1}, *aadA2*, *sul1*, *tet*(A) (n=1) STR, SUL, TET/*aadA2*, *sul1*, *tet*(A) (n=8) Susceptible (n=5)	*merA* + *aadA2*, *sul1, tet*(A)[f] - Chr (n=1) *merA* – ND (n=8) (-) (n=5)	32-36/1-2 (n=3)	0.16-0.25/ND (n=3)
	ND (n=2)	2002, 2010; S	Food/P (n=1); Human/H (n=1)	STR, SUL/*aadA2*, *sul1* (n=1) TET/(-) (n=1)	(-) (n=2)		
Essen (n=1)	ND (n=1)	2003; C	Environment/W (n=1)	Susceptible (n=1)	(-) (n=1)	32/24 (n=1)	0.125/ND (n=1)
Farsta (n=1)	ND (n=1)	2002; S	Food/Pt (n=1)	NAL, CIP^{DS}/ND (n=1)	(-) (n=1)	36/2 (n=1)	0.125/ND (n=1)
Freiburg (n=1)	ND (n=1)	2010; N	Human/C (n=1)	Susceptible (n=1)	(-) (n=1)	36/2 (n=1)	0.125/ND (n=1)
Gaillac (n=1)	ND (n=1)	2002; S	Food/OT (n=1)	Susceptible (n=1)	(-) (n=1)	36/2 (n=1)	0.125/ND (n=1)
Give (n=4)	ND (n=2)	2002-2003; N	Food/P (n=2)	Susceptible (n=2)	(-) (n=2)	36/2 (n=1)	0.16/ND (n=1)
	GA (n=2)	2008; N	Animal production/Pi (n=2)	Susceptible (n=2)	(-) (n=2)		
Gloucester (n=1)	ND (n=1)	2004; S	Food/P (n=1)	Susceptible (n=1)	(-) (n=1)	32/2 (n=1)	0.125/ND (n=1)
Goldcoast (n=2)	R (n=1)/ST358	2003; N	Human/H (n=1)	AMX, CHL, GEN, STR, SUL, TET, TMP/bla_{TEM-1}, *catA, aadA2*, *sul1-sul2*, *tet*(A), *dfrA12* (n=1)	*merA* + bla_{TEM-1}, *aadA2, sul1-sul2, tet*(A), *dfrA12*[f] – PL (95, UN) (n=1)	28-36/2 (n=2)	0.16-0.25 (n=2)/0.16 (n=1)
	ND (n=1)/ST358	2002; N	Food/P (n=1)	Susceptible (n=1)	(-) (n=1)		
Guerin (n=1)	GB (n=1)/ST508	2011; N	Animal production/A (n=1)	Susceptible (n=1)	(-) (n=1)	36/2 (n=1)	0.125/ND (n=1)
Hadar (n=4)	ND (n=4)	2002; N, C, S	Food/Pt (n=4)	AMX, STR, TET, NAL, CIP^{DS}/bla_{TEM-1}, *strA*, *tet*(A) (n=1) TET, STR, NAL, CIP^{DS}/*tet*(A), *strA-*	(-) (n=4)	32/8 (n=1)	0.16/ND (n=1)

Serotype (no. isolates)	**"Clone designation" and PFGE-type** (no. isolates)/**ST**[a]	**Isolation date; region**[b]	**Source/sample** (no. isolates)[c]	**Antibiotic resistance profile/genes** (no. isolates)[d]	**Other metal tolerance genes + co-location with antibiotic resistance genes - Chr or PL (Kb, Inc)** (no. isolates)[e]	**MIC_{range} $CuSO_4$ Aerobic/anaerobic (mM)** (no. isolates)	**MIC_{range} $AgNO_3$ Aerobic/with Ag contact (mM)** (no. isolates)
				strB (n=1) AMX, NAL/bla_{TEM-1} (n=1) NAL, CIP^{DS}/ND (n=1)			
Heidelberg (n=2)	F (n=1)/ST15	2002; S	Food/Pt (n=1)	AMX, SUL, TET, TMP/bla_{TEM-1}, *sul1*, *tet*(A), *dfrA1, aadA1* (n=1)	*merA* + bla_{TEM-1}, *sul1*, *tet*(A), *dfrA1, aadA1*[f] – PL (120, P) (n=1)	28-32/1-2 (n=2)	0.16-0.25/ND (n=2)
	ND (n=1)	2003; N	Human/H (n=1)	Susceptible (n=1)	(-) (n=1)		
Houston (n=2)	P (n=2)/ST48	2003-2004; N	Food/B (n=1); Human/C (n=1)	AMX, KAN, STR, SUL, TET, TMP/bla_{TEM-1}, *aphA1, aadA2*, *sul1-sul2*, *tet*(A), *dfrA12* (n=2)	*merA* + bla_{TEM-1}, *aphA1, aadA2, sul1-sul2*, *tet*(A), *dfrA12*[f] – PL (95, UN) (n=1) *merA* – ND (n=1)	32-36/1-4 (n=2)	0.16 (n=2)/0.125 (n=1)
Indiana (n=1)	ND (n=1)	2011; S	Environment/W (n=1)	Susceptible (n=1)	(-) (n=1)	32/2 (n=1)	0.125/ND (n=1)
Infantis (n=3)	E (n=1)/ST32	2003; S	Food/P (n=1)	STR, SUL, TET, NAL, CIP^{DS}/*aadA1*, *sul1*, *tet*(A) (n=1)	*merA* + *aadA1, sul1, tet*(A)[f] – PL (290, P) (n=1)	32-36/1-4 (n=2)	0.16/ND (n=2)
	ND (n=2)	2002-2003; N	Human/C (n=1) and H (n=1)	Susceptible (n=2)	(-) (n=2)		
Istambul (n=1)	ND (n=1)	2002; S	Food/Pt (n=1)	NAL, CIP^{DS}/ND (n=1)	(-) (n=1)	32/2 (n=1)	0.125/ND (n=1)
Kubacha (n=1)	ND (n=1)	2002; Unknown	Food/P (n=1)	Susceptible (n=1)	(-) (n=1)	36/2 (n=1)	0.125/ND (n=1)
Linguere (n=1)	LA (n=1)/ST508	2011; C	Animal production/A (n=1)	Susceptible (n=1)	(-) (n=1)	32/1 (n=1)	0.125/ND (n=1)
Livingstone (n=1)	ND (n=1)	2011; S	Food/U (n=1)	Susceptible (n=1)	(-) (n=1)	32/2 (n=1)	0.125/ND (n=1)
Marshall (n=1)	ND (n=1)	2010; S	Human/H (n=1)	Susceptible (n=1)	(-) (n=1)	36/2 (n=1)	0.125/ND (n=1)
Mbandaka (n=5)	ND (n=2)	2002, 2010; C	Food/Pt or U (n=2)	Susceptible (n=1) Susceptible (n=1)	(-) (n=2)	32/2 (n=1)	0.125/ND (n=1)
	MA (n=1)	2008; N	Animal production/Pi (n=1)	STR/*strA-strB* (n=1)	(-) (n=1)		
	MB (n=2)	2008; N	Animal production /Pi (n=2)	Susceptible (n=2)	(-) (n=2)		
Muenchen (n=3)	A (n=1)/ST118	2011; C	Animal production/A (n=1)	STR/(-) (n=1)	(-) (n=1)	32/2 (n=1)	0.125/ND (n=1)
	ND (n=2)	2003, 2010; S	Human/H (n=2)	Susceptible (n=2)	(-) (n=2)	32/2 (n=1)	0.125/ND (n=1)
Newport (n=1)	ND (n=1)	2003; N	Human/H (n=1)	Susceptible (n=1)	(-) (n=1)	36/2 (n=1)	0.125/ND (n=1)
Ohio (n=1)	ND (n=1)	2011; S	Human/H (n=1)	AMX, KAN, STR, SUL, TET, NAL, CIP^{DS}/bla_{TEM}, *aphA1*, *strA*, *sul2*, *tet*(A) (n=1)	(-) (n=1)	36/2 (n=1)	0.125/ND (n=1)
Ramsey (n=1)	ND (n=1)	2011; S	Human/H (n=1)	Susceptible (n=1)	(-) (n=1)	36/8 (n=1)	0.125/ND (n=1)

Serotype (no. isolates)	**"Clone designation" and PFGE-type** (no. isolates)**/ST**[a]	**Isolation date; region**[b]	**Source/sample** (no. isolates)[c]	**Antibiotic resistance profile/genes** (no. isolates)[d]	**Other metal tolerance genes + co-location with antibiotic resistance genes - Chr or PL (Kb, Inc)** (no. isolates)[e]	**MIC_{range} $CuSO_4$ Aerobic/anaerobic (mM)** (no. isolates)	**MIC_{range} $AgNO_3$ Aerobic/with Ag contact (mM)** (no. isolates)
Saintpaul (n=3)	I (n=1)/ST27	2002; S, Unknown	Food/Pt (n=3)	AMX, CHL, STR, SUL, TET, TMP/*bla*$_{TEM-1}$, *catA*, *aadA1*, *sul1-sul2*, *tet*(A), *dfrA1* (n=3)	*merA* - ND (n=3)	32/1 (n=3)	0.16 (n=3)/0.125 (n=1)
	H (n=2)/ST27						
Stanley (n=2)	ND (n=2)	2011; N	Human/H (n=2)	Susceptible (n=2)	(-) (n=2)	36/2 (n=1)	0.125/ND (n=1)
Teddington (n=1)	ND (n=1)	2002; N	Food/Pt (n=1)	Susceptible (n=1)	(-) (n=1)	32/2 (n=1)	0.125/ND (n=1)
Tomphson (n=1)	ND (n=1)	2010; S	Human/H (n=1)	STR/(-) (n=1)	(-) (n=1)	28/1 (n=1)	0.125/ND (n=1)
Uganda (n=1)	ND (n=1)	2002; N	Food/Pt (n=1)	Susceptible (n=1)	(-) (n=1)	32/2 (n=1)	0.125/ND (n=1)
Urbana (n=1)	ND (n=1)	2010; S	Human/H (n=1)	Susceptible (n=1)	(-) (n=1)	36/2 (n=1)	0.125/ND (n=1)
Virchow (n=4)	ND (n=4)	2002-2003; N	Animal production/Pi (n=3); Human/H (n=1)	STR/(-) (n=1) NAL, CIP^{DS}/ND (n=1) Susceptible (n=2)	(-) (n=4)	36/1 (n=1)	0.125/ND (n=1)
II 4,5:b:- (n=1)	ND (n=1)	2010; S	Food/U (n=1)	STR/(-) (n=1)	(-) (n=1)	32/28 (n=1)	0.125/ND (n=1)
II 42:b:enxz15 (n=1)	ND (n=1)	2003; N	Environment/WR (n=1)	Susceptible (n=1)	(-) (n=1)	32/12 (n=1)	0.125/ND (n=1)
II O4,12:z:1,7 (n=1)	ND (n=1)	2003; N	Food/U (n=1)	Susceptible (n=1)	(-) (n=1)	36/2 (n=1)	0.125/ND (n=1)
IIIa 48:z4 (n=1)	ND (n=1)	2011; S	Environment/W (n=1)	Susceptible (n=1)	(-) (n=1)	36/1 (n=1)	0.125/ND (n=1)
IIIb 50:i:z53 (n=1)	ND (n=1)	2010; S	Human/H (n=1)	STR/(-) (n=1)	(-) (n=1)	28/1 (n=1)	0.125/ND (n=1)

AMX, amoxicillin; CTX, cefotaxime; CHL, chloramphenicol; CIP, ciprofloxacin; CIP^{DS}, decreased susceptibility to ciprofloxacin, GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TMP, trimethoprim; Chr, chromosome; PL, plasmid; ND, not determined; UN, untypable.

[a]"Clone designation" was adopted only when has been previously published. PFGE-types were designated by capital letters, including previously described[1,21-24] or firstly designated in this study.

[b]N, north; C, center; S, south; I, islands (Azores).

[c]A, aquacultures environment; B, beef; C, community; Cw, cow; H, hospitals patients; OT, other type of food; P, pork; Pt, poultry; Pi, pigs and piggeries environment; Pt, poultry; Q, quail; U, unknown; W, bathing or drinking water; WR, river water.

Table S1. *Continued*

[d](-), Absence of all antibiotic resistance genes searched for. Isolates resistant to three or more antibiotics from different families were considered multidrug-resistant.

[e](-), Absence of all metal tolerance genes searched for. Underlined metal tolerance or antibiotic resistance genes were also detected in transconjugants.

[f]In particular serotypes/clonal lineages *merA*, *arsB* and/or *terF* were in the same genetic platform that the genes belonging to conventional class 1 integrons [1000bp (*aadA1*) in *S*. Infantis; 1000bp (*aadA2*) in *S*. Derby; 1600bp (*aadB-aadA2*) in *S*. Bovismorbificans; 1700bp (*dfrA1-aadA1*) in *S*. Enteritidis and *S*. Heidelberg; 2000bp (bla_{OXA-30}-*aadA1*) in *S*. Typhimurium "OXA-30-producing" clone; 2000bp (*dfrA12-orfF-aadA2*) in *S*. Goldcoast and *S*. Houston] or to atypical *sul3*-type I integrons 7085bp (5'CS-*dfrA12-orfF-aadA2-cmlA1-aadA1-qacH*-IS440-*sul3*) in *S*. Typhimurium "DT104"/Q clone and *S*. Anatum].[21-24]

Table S2. Primers used in PCR assays to search acquired metal tolerance genes

Operon or cluster	Target Gene	Gene coding for	Primer	Primer sequence (5'-3')[a]	Amplicon size (bp)	Annealing temperature (ºC)	Nucleotide position (GenBank accession no.)	Reference
sil	*silA*	Silver inner-membrane proton/cation antiporter	silA_Fw silA_Rv	GCAAGACCGGTAAAGCAGAG CCTGCCAGTACAGGAACCAT	936	59	8936-8955 9871-9852 (**AF067954.1**)	[6]
	silC	Outer membrane protein	silC_4090_F silC_4470_R	CGGGCTGGCGHAMCTTTTTTG CCAGTTGCTGRYTGAAATARC	381	60	4090-4110 4470-4450 (**BX664015.1**)	This study
	silE	Silver/copper periplasmic binding protein	silE_1105_F silE_1368_R	GTTCGTCATGGTYTCATGAGC GTACTYCCCCGGACATCACTAATT	264	62	1105-1125 1368-1345 (**AF067954.1**)	[6]
	silP	P-type cation ATPase	silP_11882_F silP_12405_R	GGCGATAAGCTCCGCATCAGA TCCACTTTTTCAAGACGCTCA	524	60	11882-11902 12405-12385 (**BX664015.1**)	[54]
	silR	Transcriptional regulator responder	silR_3244_F silR_3572_R	CCCTGATGGCGAAKGAAGAA AACGGCTGGGATATCRTSCG	329	62	3244-3263 3572-3553 (**BX664015.1**)	[54]
	silS	Membrane kinase sensor	silS_2279_F silS_3019_R	GCGGGTAAARACATCCTCAAT CTGACCTTTTTTATCAGCCTG	741	60	2279-2299 3019-2999 (**BX664015.1**)	[54]
pco	*pcoA*	Multicopper oxidase	pcoA_978_F pcoA_1481_R	CTCGCGGATGTCAGTGGCTACACCT ATCCGGAAGGTCAGCACCGTCCATAGAC	504	60	978-1002 1481-1454 (**X83541.1**)	[6]
	pcoD	Copper inner membrane pump	pcoD_F pcoD_R	CTGGCCACACTTGCCTGGGG CACGCTACGGCGCCCAGAAT	500	55	3801-3820 4300-4281 (**X83541.1**)	[6]
	pcoE	Periplasmic chaperone	pcoE_6585_F pcoE_7057_R	CCTGGTTCCTGGAGTGATGA TGACCATTATTCGCCTTCT	473	58	6585-6604 7057-7039 (**X83541.1**)	This study
	pcoR	Regulator of *pcoABCD* cluster	pcoR_4460_F pcoR_4928_R	AGGCTATCAGGCCGATCTCT CCAGACCAGGGACGAGATAA	469	58	4460-4478 4928-4909 (**X83541.1**)	This study

Table S2. *Continued*

Operon or cluster	Target Gene	Gene coding for	Primer	Primer sequence (5'-3')[a]	Amplicon size (bp)	Annealing temperature (ºC)	Nucleotide position (GenBank accession no.)	Reference
	pcoS	Sensor of *pcoABCD* cluster	pcoS_5182_F pcoS_6236_R	TAATCAGGACCGCGATTTTC CACTGTCACGCTCAAGGTGT	1055	63	5182-5201 6236-6217 (**X83541.1**)	This study
mer	*merA*	Mercuric reductase	merA_1F merA_5R	ACCATCGGCGGCACCTGCGT ACCATCGTCAGGTAGGGGAAC	1238	65	2140-2159 3377-3357 (**K03089.1**)	[43]
ars	*arsB*	Arsenite transmembrane pump	arsB_Fw arsB_Rv	AGTGAAAGACAGACGAAGCG GGCAGATAGTGTGGAATGCG	1136	60	159735-159754 160870-160851 (**BX664015.1**)	[55]
ter	*terF*	Tellurite resistance protein	terF_Fw1 terF_Rv	ATAGCACTGGATCGTGTTCC TTCATCGATCCACGGTCTG	990	60	80174-80193 81163-81145 (**BX664015.1**)	This study

[a]H = A, C or T; K = G or T; M = A or C; R = A or G; S = G or C; Y = C or T.

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Table S3. Dispersion of *sil±pco* gene clusters and their genetic environments in *Salmonella enterica* genomes available at GenBank database

Cu/Ag cluster[a]	Serotype (no. isolates)	Strain name(s)/ST (no. isolates)[b]	Country/Year(s) of isolation	Source	Genetic element associated with *sil±pco* clusters[a,d]		Insertion site related with the genetic element (no. isolates)[e]	GenBank accession number or reference
					Tn*6230*	Tn*6230*-partial		
sil	Typhimurium (n=5)	34502, YU39, CH9, CH44/ST213 (n=2)	NR; Canada/2000-2001; Mexico/2005	Swine, children blood		+	Other (n=4)	AUQT01000028.1, CP011429.1, CQHB01000038.1, CQHS01000024.1
		35423/ST213 (n=1)	NR	Cattle			NA (n=1)	([f])
sil+pco	4,12:i:- (n=8)	CVM N48691, CVM N50420, CVM N50445, CVM N51240, CVM N51270, CVM N51287, CVM N51306	USA/2013	Pork chops, ground turkey		+	tRNA-Phe (n=7)	LHKM01000014.1, LHLF01000008.1, LHMA01000018.1, LHMF01000007.1, LHNH01000009.1, LHNW01000011.1, LHOM01000029.1
		STM3910	Italy/2011	Human faeces			NA (n=1)	([f])
	I 8,20:-:z6 (n=1)	CVM N43471	USA/2013	Chicken breast			NA (n=1)	([f])
	Agona (n=2)	CVM N47726	USA/2013	Ground turkey	+		*yhiN* gene (n=1)	LHJT01000022.1
		21.H.10/ST13 (n=1)	Germany/2010	Human faeces			NA (n=1)	([f])
	Anatum (n=3)	CFSAN024764	Chile/2009	Human	+		*yhiN* gene (n=1)	JWQO01000019.1
		CVM N44698	USA/2013	Ground turkey	+		Other (n=1)	LHFG01000020.1
		CVM N44699	USA/2013	Ground turkey			NA (n=1)	([f])

Cu/Ag cluster[a]	Serotype (no. isolates)	Strain name(s)/ST (no. isolates)[b]	Country/Year(s) of isolation	Source	Genetic element associated with *sil±pco* clusters[a,d]		Insertion site related with the genetic element (no. isolates)[e]	GenBank accession number or reference
					Tn*6230*	Tn*6230*-partial		
	Bovismorbificans (n=1)	499208 (n=1)	UK/1970-1980	Alpaca	+		Other (n=1)	CSTH01000028.1
	Brandenburg (n=1)	CVM N45949 (n=1)	USA/2013	Chicken breast	+		Other (n=1)	LHHJ01000009.1
	Cubana (n=4)	CFSAN002050, CFSAN001083, CVM42234/ST286 (n=2)	Philippines/1972; USA/2012	Fresh alfalfa sprouts, dessicated coconut, chick feed		+	tRNA-Phe (n=3)	CP006055.1, APAG01000439.1, ATEU01000022.1
		76814	USA/2004	Swine			NA (n=1)	([f])
	Derby (n=1)	CVM N51280	USA/2013	Pork chops			NA (n=1)	([f])
	Havana (n=1)	CFSAN024771	Chile/2012	Garuma gull			NA (n=1)	([f])
	Heidelberg (n=27)	SH111_227, 24393, SARA 39, CVM N44695, CVM N44710, CVM N45397, CVM N45951, CVM N45956, CVM N46812, CVM N48578, CVM N48699, CVM N50450, CVM N51244	USA/2001, 2003, 2013	Bovine, turkey intestines, human, chicken wings, pork chops, ground turkey, ground beef, chicken breast	+		Other (n=13)	[36, 56], LHFD01000022.1, LHFQ01000012.1, LHGB01000032.1, LHHL01000045.1, LHHQ01000047.1, LHHU01000028.1, LHJY01000019.1, LHKU01000049.1, LHME01000058.1, LHMI01000035.1
		CFSAN002064, CFSAN002069, CVM N45958, CVM N46811,	USA/2012, 2013	Human stool, chicken sample, chicken breast,	+		*yhiN* gene (n=5)	CP005995.1, CP005390.2, LHHS01000023.1,

Cu/Ag cluster[a]	Serotype (no. isolates)	Strain name(s)/ST (no. isolates)[b]	Country/Year(s) of isolation	Source	Genetic element associated with *sil±pco* clusters[a,d]		Insertion site related with the genetic element (no. isolates)[e]	GenBank accession number or reference
					Tn*6230*	Tn*6230*-partial		
		CVM N50449/ST15 (n=1)		chicken wings				LHHT01000004.1, LHMD01000005.1
		20752, 32507, 24359, N15757, N30678, 41565, CVM N44707, CVM N51243, CVM N51984	USA/2002, 2003, 2007, 2011, 2013	Turkey cecal tonsil, ground turkey, turkey Intestines, chicken breast, pork chops			NA (n=9)	([f])
	Infantis (n=3)	CVM N44705, Sal147, Sal280	Portugal/2011, 2012; USA/2013	Chicken breast, food sample			NA (n=3)	([f])
	Kentucky (n=21)	CVM N47723, CVM N45934, CVM N45939, CVM N45944, CVM N46849, CVM N48705, CVM N48710	USA/2013	Ground turkey, chicken breast	+		Other (n=1), NA (n=6)	LHJQ01000057.1, LHGX01000055.1, LHHC01000025.1, LHHH01000045.1, LHIU01000057.1, LINU01000071.1, LHLB01000019.1
		N312, 20793, CVM N43447, CVM N47720, CVM N50429, CVM N51981/ST152 (n=2)	USA/2002, 2004, 2013	Chicken breast		+	Other (n=6)	AUQI01000016.1, AUQJ01000021.1, LHDL01000020.1, LHJO01000031.1, LHLN01000030.1, LHOT01000028.1
		SALC-205-3, CVM N44693, CVM N45412, CVM N45937, CVM N47729, CVM N47730, CVM N48687, CVM N51241	USA/2013; Canada/2004	Chicken breast, chicken fecal material, chicken wings			NA (n=8)	([f])

Cu/Ag cluster[a]	Serotype (no. isolates)	Strain name(s)/ST (no. isolates)[b]	Country/Year(s) of isolation	Source	Genetic element associated with *sil±pco* clusters[a,d]		Insertion site related with the genetic element (no. isolates)[e]	GenBank accession number or reference
					Tn*6230*	Tn*6230*-partial		
	Liverpool (n=1)	CVM N50444	USA/2013	Ground turkey	+		Other (n=1)	LHLZ01000022.1
	Mbandaka (n=1)	CVM N51302	USA/2013	Chicken breast	+		*yhiN* gene (n=1)	LHOI01000026.1
	Montevideo (n=45)	S5-403, 315996572, 609458-1, 495297-1, 495297-3, 495297-4, 515920-1, 515920-2, 609460, NC_MB110209-0054, OH_2009072675, CASC_09SCPH15965, 19N, 81038-01, MD_MDA09249507, 414877, 366867, 413180, 446600, 556152 , 556150-1, MB101509-0077, MB102109-0047, MB111609-0052, 2009083312, 2009085258, 315731156, IA_2009159199, IA_2010008282, IA_2010008283, IA_2010008284, IA_2010008287, CT_02035278, CT_02035318, 80959-06, CT_02035320, CT_02035321, IA_2010008286, 507440-20, MB110209-0055,	USA/NR, 2006, 2007, 2009, 2010, 2013	Pistachio[c], black peper[c], human stool, chicken[c], hearts of romaine[c], human sample, mozzarella[c], perch[c], sea trout[c], king fish[c], red pepper flakes[c], lunch meat[c], environmental isolate[c], environmental swab[c], red pepper, chcken breast	+		*yhiN* gene (n=42)	[40], AESH01000033.1, AETF01000076.1, AESI01000042.1, AESJ01000015.1, AESK01000007.1, AESL01000025.1, AESM01000016.1, AETH01000090.1, AESO01000068.1, AESP01000033.1, AESQ01000058.1, AESV01000032.1, AESZ01000024.1, AETA01000018.1, AETB01000005.1, AETC01000011.1, AETD01000010.1, AETE01000043.1, AETJ01000070.1, AETG01000080.1, AETK01000018.1, AETL01000127.1, AETN01000032.1, AETO01000025.1, AETP01000034.1, AETQ01000024.1,

Cu/Ag cluster[a]	Serotype (no. isolates)	Strain name(s)/ST (no. isolates)[b]	Country/Year(s) of isolation	Source	Genetic element associated with *sil±pco* clusters[a,d]		Insertion site related with the genetic element (no. isolates)[e]	GenBank accession number or reference
					Tn*6230*	Tn*6230*-partial		
		FCC0146, CVM N51288/ST316 (n=38); 4441 H/ST138 (n=1)						AETR01000099.1, AETS01000072.1, AETT01000046.1, AETU01000051.1, AETW01000065.1, AHHS01000110.1, AHHT01000019.1, AHHR01000012.1, AHHU01000003.1, AHHR01000012.1, AHIK01000009.1, CP007530.1, AETM01000157.1, JSWT01000001.1, LHNX01000007.1, AESY01000016.1
		IA_2010008285, CT_02035327/ST316 (n=2)	USA/NR, 2010	Lunch meat[c], environmental isolate[c]			NA (n=2)	([f])
	Ohio (n=1)	CFSAN001079/ST329 (n=1)	USA/NR	Pork sausage		+	tRNA-Phe (n=1)	APAK01000322.1
	Rissen (n=1)	150	USA/2009	Food from white pepper outbreak		+	Other (n=1)	AHUI01000058.1
	Schwarzengrund (n=15)	CFSAN003382, CVM N48682, CVM N51311	USA/2011, 2013	Creek water, ground turkey, chicken breast	+		*yhiN* gene (n=3)	LARZ01000012.1, LHKE01000010.1, LHOP01000021.1
		CVM N43459, CVM	USA/2013	Chicken wings,		+	Other (n=6)	LHDW01000030.1,

Cu/Ag cluster[a]	Serotype (no. isolates)	Strain name(s)/ST (no. isolates)[b]	Country/Year(s) of isolation	Source	Genetic element associated with *sil±pco* clusters[a,d]		Insertion site related with the genetic element (no. isolates)[e]	GenBank accession number or reference
					Tn*6230*	Tn*6230*-partial		
		N43479, CVM N44700, CVM N50434, CVM N51257, CVM N51289		chicken breast				LHEJ01000002.1, LHFI01000042.1, LHLQ01000036.1, LHMV01000011.1, LHNY01000009.1
		CVM N45952, CVM N47711, CVM N47712, CVM N51259, CVM N51267	USA/2013	Ground turkey, ground breast, chicken breast		+	tRNA-Phe (n=5)	LHHM01000008.1, LHJG01000015.1, LINN01000004.1, LHMX01000017.1, LHNF01000001.1
		CVM N47722	USA/2013	Ground turkey	+		Other (n=1)	LINO01000043.1
	Senftenberg (n=21)	A4-543, SS209, NC_MB012510-0038, 604314, 423984-2, CFSAN024718, CFSAN024719, CFSAN024720, CFSAN024721, CFSAN024722, CFSAN024723, CFSAN024724, NCTC10384, CVM N43462, CVM N51274, CVM N51312/ST14 (n=4)	NR/1964; USA/NR, 2005, 2006, 2010, 2013; France/NR; Chile/2009-2013	Cattle, gastroenteritis, human stool, black peper, salame-soprasetta, kelp gull, human sample, faeces, chicken wings, ground turkey	+		*yhiN* gene (n=16)	[40], CAGQ01000054.1, AYEJ01000033.1, AOXW01000018.1, AOYP01000072.1, JWRE01000006.1, JWRD01000007.1, JWRC01000002.1, JWRB01000009.1, JWRA01000005.1, JWQZ01000006.1, JWQY01000030.1, LN868943.1, LHDZ01000008.1, LHNK01000004.1, LHOQ01000023.1

Cu/Ag cluster[a]	Serotype (no. isolates)	Strain name(s)/ST (no. isolates)[b]	Country/Year(s) of isolation	Source	Genetic element associated with *sil±pco* clusters[a,d]		Insertion site related with the genetic element (no. isolates)[e]	GenBank accession number or reference
					Tn*6230*	Tn*6230*-partial		
		CVM N51281	USA/2013	Ground turkey	+		Other (n=1)	LHNQ01000022.1
		ATCC 8400/ST290 (n=1)	USA/NR	NR		+	tRNA-Phe (n=1)	AOXU01000003.1
		423984-1, 316235162, 361154004/ST14 (n=3)	USA/NR, 2009	Environmental swab[c], pistachios, shelled pistachios			NA (n=3)	([f])
	Typhimurium (n=10)	S7, S15, S23, ST4020, ST4024, Sal368	Denmark/NR; Hong Kong/2007; Portugal/ 2012	Food, copper feed pigs, human blood		+	tRNA-Phe (n=6)	[41], LFGX01000049.1, JRYU01000005.1, JRYU01000005.1
		MG101, ST2533, ST2286, FSAN236CA	USA/1973; Hong Kong/2006; Colombia/2012	Human burn, human stool; animal feed, raw chicken carcass at slaughterhouse	+		Other (n=2), NA (n=2)	[57, 58], JRZP01000030.1, JRZR01000027.1, LIKP01000046.1
	Tennessee (n=2)	TXSC_TXSC08-19/ST319 (n=1)	USA/2004	Fishmeal	+		*yhiN* gene (n=1)	CP007505.1
		TXSC_TXSC08-21/ST319 (n=1)	New Zealand/2004	Meat and bone meal, lamb-based			NA (n=1)	([f])
	Worthington (n=4)	CVM N45406, CVM N45931, CVM N46835, CVM N51304	USA/2013	Chicken breast, ground turkey		+	tRNA-Phe (n=4)	LHGK01000008.1, LHGV01000004.1, LHIL01000001.1, LHOK01000015.1

NR, not reported

Table S3. *Continued*

[a]Analysis of *sil*±*pco* gene clusters dispersion as well as their genetic environments was performed in completed *Salmonella* genomes, plasmid sequences or contigs (when visualization of the genetic environment was possible) available for Microbial Genome BLAST® (http://blast.ncbi.nlm.nih.gov/Blast.cgi; accessed 19/10/2015). The sequences were searched using amino acidic sequences (blastp) of *pcoA* and *pcoD* from pRJ1004 (accession no. CAA58528.1)[28] and *silA* and *silE* from pMG101 (accession no. AAD11749.1)[58] as query, and those with a minimum of 97% amino acidic identity were selected. What we consider in the manuscript as a common *sil*+*pco* genetic module corresponds to part of Tn*6230* (*sil*+*pco* gene clusters and genes coding for a hypothetical protein and a total/partial endonuclease; e.g. GenBank accession number AOXW01000018.1 from 180790 bp to 157014 bp), was based on the synteny of those genes and not in the differences of the intergenic regions (variability observed between ≈ 21 and 24 Kb), or small nucleotide variability within each gene.

[b]Number of isolates with sequence types (STs) previously assigned, according to the *Salmonella* MLST scheme (http://mlst.warwick.ac.uk/mlst/dbs/Senterica) and available in NCBI (http://www.ncbi.nlm.nih.gov/) or PATRIC (https://www.patricbrc.org/portal/portal/patric/Home) databases.

[c]Isolates with different source information among databases. The information here considered was that available in BioSample NCBI (http://www.ncbi.nlm.nih.gov/biosample/).

[d]Two types of genetic environments were found associated with the common *sil*+*pco* module and marked with "+" when they are present in the analysed strains (n=174): "Tn*6230*", when the typical proteins (TnsABC) involved in transposition of these genetic elements are present or "Tn*6230*-partial" corresponding to the common module but without the TnsABC proteins. The absence of "+" means that it was not possible to evaluate if the genetic element was complete or partial due to the interruption of the contig.

[e]Insertion sites related to Tn*6230* or Tn*6230*-partial genetic elements: "*yhiN* gene" – common insertion site of Tn*6230*; "tRNA-phe" – consists of the common tRNA-Phe + xerD recombinase + relaxase + DNA helicase region proximal to Tn*6230*-partial element; "Others" – Tn*6230* or Tn*6230*-partial were found in other insertion sites beside *yhiN* gene or tRNA-phe region; "NA", not applicable when it was not possible to evaluate the insertion site of the genetic element due to the interruption of the contig.

[f]GenBank accession numbers of isolates with interrupted contigs. *Salmonella* serotypes with *sil* cluster: Typhimurium (AUQU01000026.1) and with *sil*+*pco* clusters: 4,12:i:- (LJJK01000035.1), I 8,20:-:z6 (LHEE01000083.1), Agona (CASU01000164.1), Anatum (LHFH01000016.1), Cubana (AZGR01000012.1), Derby (LHNP01000052.1), Havana (JWQI01000012.1), Heidelberg

Table S3. *Continued*

(AMNM01000010.1, AMNL01000012.1, AMNP01000117.1, AMND01000026.1, AMNK01000100.1, AJHA01000116.1, LHFN01000150.1, LIOA01000049.1, LHOW01000029.1), Infantis (LHFL01000049.1, LFGU01000369.1, LFGW01000003.1), Kentucky (Dhanani *et al*., 2015, LIMQ01000067.1, LHGN01000037.1, LHHA01000088.1, LHJV01000024.1, LHJW01000021.1, LINR01000077.1, LHMG01000006.1), Montevideo (AETV01000009.1, AHHW01000039.1), Senftenberg (AYDP01000247.1, AOYU01000007.1, AYDO01000054.1), Tennessee (AOXQ01000059.1, AOXQ01000257.1).

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Salmonella enterica serotype Bovismorbificans, a new host for CTX-M-9

Patrícia Antunes[1,2], Joana Mourão[2], Tatiana Alves[2], Joana Campos[2], Carla Novais[2], Ângela Novais[2] and Luísa Peixe[2]*

[1] Faculdade de Ciências da Nutrição e Alimentação, Universidade do Porto, Porto, Portugal; [2] REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal.

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Table 1
Features of the carbapenem-resistant isolates and patient characteristics.

Patient	Hospitalisation unit	Isolate	Site of isolation	Carbapenemase	Associated β-lactamases	MLST type	MICs of carbapenems		
							IPM	MER	ERT
1	Oncology	*Klebsiella pneumoniae*	Stool	OXA-48	None	ST14	2	0.75	1.5
2	Outpatient	*Escherichia coli*	Pus	OXA-48	CTX-M-14, TEM-1, SHV-12	ST38	0.75	0.25	1.5
3	Oncology	*E. coli*	Blood	OXA-48 + VIM-1	None	ST101	32	>32	>32
4	Bone marrow transplantation unit	*K. pneumoniae*	Throat swab	VIM-1	CTX-M-14, TEM, SHV-12	ST17	1.5	0.5	0.75
5	Oncology	*K. pneumoniae*	Urine	VIM-1	CTX-M-15	ST101	32	>32	>32
6	Oncology	*Enterobacter cloacae*	Pus	VIM-1	None	N/D	8	0.5	0.25
7	Paediatric oncology	*E. cloacae*	Peritoneal fluid	VIM-1	SHV-12	N/D	32	32	16
8	Oncology	*E. cloacae*	Pus	VIM-1	None	N/D	1	0.5	1
9	Oncology	*E. coli*	Pus	VIM-1	TEM-1, SHV-12	ST23	1	0.5	0.5
10	Paediatric oncology	*E. cloacae*	Blood	VIM-1	CTX-M-9, TEM-1, SHV-11	N/D	1.5	0.5	0.5

MLST, multilocus sequencing typing; MIC, minimum inhibitory concentration; IPM, imipenem; MER, meropenem; ERT, ertapenem; N/D, not determinable.

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Laurent Poirel*
Service de Bactériologie-Virologie, INSERM U914 'Emerging Resistance to Antibiotics', Hôpital de Bicêtre, Assistance Publique/Hôpitaux de Paris, Faculté de Médecine Paris Sud, K.-Bicêtre, France

Mohammed O. Abdelaziz
Department of Microbiology and Immunology, Faculty of Pharmacy, Helwan University, Cairo, Egypt

Sandrine Bernabeu
Patrice Nordmann
Service de Bactériologie-Virologie, INSERM U914 'Emerging Resistance to Antibiotics', Hôpital de Bicêtre, Assistance Publique/Hôpitaux de Paris, Faculté de Médecine Paris Sud, K.-Bicêtre, France

* Corresponding author. Present address: Service de Bactériologie-Virologie, Hôpital de Bicêtre, 78 rue du Général Leclerc, 94275 Le Kremlin-Bicêtre Cedex, France.
Tel.: +33 1 45 21 3632; fax: +33 1 45 21 63 40.
E-mail address: laurent.poirel@bct.aphp.fr (L. Poirel)

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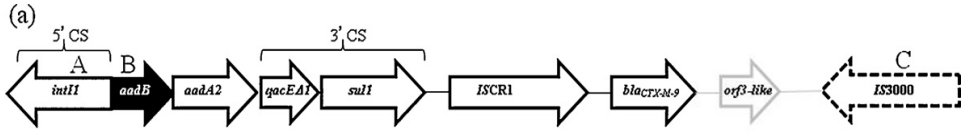
Salmonella enterica serotype Bovismorbificans, a new host for CTX-M-9

Sir,

The spread of non-typhoidal *Salmonella* resistant to broad-spectrum cephalosporins constitutes an emerging threat worldwide, although their distribution is uneven in different geographical regions and ecological niches [1]. Although production of AmpC-type enzymes, mainly CMY-2, is rising in Europe, extended-spectrum β-lactamases (ESBLs) are still the most associated with resistance to cephalosporins [1]. Different food-producing animals and food carry ESBL-producing bacteria, with CTX-M being the most frequent family [1]. The CTX-M-9 enzyme is one of the most frequently found in *Salmonella* both of human and animal origin [1], linked to the clonal spread of a particular *Salmonella* serotype (*Salmonella enterica* serotype Virchow) and food-producing animals (poultry) in neighbouring countries (Spain and France) [1,2]. In this study, we describe the molecular characterisation of the first ESBL-producing *Salmonella* in Portugal, consisting of $bla_{CTX-M-9}$ located in an In*60*-like structure and within IncHI2 plasmid, carried by an emerging human *Salmonella* serotype, *S. enterica* serotype Bovismorbificans.

Susceptibility to antibiotics as well as β-lactamase production was assayed by disc diffusion and/or Etest [Clinical and Laboratory Standards Institute (CLSI) documents M2-A10/M100-21]. Screening of genes encoding resistance to antibiotics or biocides found in the animal setting (feed, disinfectants and environmental pollution) was performed by PCR and sequencing as described

(a)



(b)

Isolate	MIC (mg/L)															Resistance phenotype/genotype to other antibiotics
	TGC	CIP	AMX	AMC	TIC	TIM	CEF	FOX	CTX	CAZ	FEP	CR	IPM	MEM	ERT	ATM	
HP507391	0.38	0.047	>256	2	>256	96	>256	6	4	0.5	1	2	0.38	0.023	0.008	1	GEN, KAN, STR, SUL, TOB/ *aadB, aadA2, sul1*
HP507391_T	0.125	0.25	>256	4	>256	128	>256	32	4	1	3	2	0.75	0.023	0.008	1.5	NAL, STR, SUL/ *aadB, aadA2, sul1*

Fig. 1. (a) Genetic context of $bla_{CTX\text{-}M\text{-}9}$ within an In*60* variant identified in a *Salmonella enterica* serotype Bovismorbificans isolate (HP507391). Differences compared with the In*60* of *S. enterica* serotype Virchow [2] are the presence of *intI1* (A), the presence of *aadB* instead of *dfrA16* (B) and the absence of insertion sequence IS*3000* (C). The discontinued arrows indicate genes not found. The grey arrows indicate genes not determined. (b) Antimicrobial susceptibility profiles and resistance genotypes of *S.* Bovismorbificans carrying $bla_{CTX\text{-}M\text{-}9}$ (HP507391) and the corresponding transconjugant (conjugation assays using *Escherichia coli* K802N) (HP507391_T). TGC, tigecycline; CIP, ciprofloxacin; AMX, amoxicillin; AMC, amoxicillin/clavulanic acid; TIC, ticarcillin; TIM, ticarcillin/clavulanic acid; CEF, cefalothin; FOX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; CR, cefpirome; IPM, imipenem; MEM, meropenem; ERT, ertapenem; ATM, aztreonam; GEN, gentamicin; KAN, kanamycin; STR, streptomycin; SUL, sulfamethoxazole; TOB, tobramycin; NAL, nalidixic acid.

previously [3]. Transferability of *bla* genes and characterisation of integron and plasmid backbones was performed by PCR, hybridisation (I-*Ceu*I/S1 nuclease) and/or sequencing [4]. The genetic environment of $bla_{CTX\text{-}M\text{-}9}$ (class 1 integron, Tn*402* derivatives) was characterised by PCR mapping based on known sequences [4]. Clonal identification was performed by multilocus sequence typing (MLST) (http://mlst.ucc.i.e./mlst/dbs/Senterica).

An *S. enterica* isolate carrying the $bla_{CTX\text{-}M\text{-}9}$ gene was isolated from the faeces of a 4-year-old boy admitted to the emergency ward of a Portuguese hospital with gastrointestinal infection in February 2011. The isolate was identified as *S.* Bovismorbificans and was assigned to sequence type 142 (ST142), previously associated with food-borne and human European isolates (http://mlst.ucc.i.e./mlst/dbs/Senterica). *Salmonella* Bovismorbificans is an unusual serotype but it is increasingly reported in Northern European countries where it has been linked to severe outbreaks traced mostly to vegetables [5]. However, it was not possible to associate the isolate reported here with consumption of a particular food products or animal contact or foreign travel. The isolate was resistant to ampicillin, cefalothin and cefotaxime, but not to ceftazidime, cefepime, aztreonam or carbapenems (Fig. 1). Resistance to gentamicin, kanamycin, tobramycin, streptomycin and sulfamethoxazole was also detected, but it was not expressed in the transconjugant (with the exception of sulfamethoxazole) irrespective of the presence of the corresponding genes (Fig. 1).

The $bla_{CTX\text{-}M\text{-}9}$ gene was associated with an unusual class 1 integron containing *aadB–aadA2* gene cassettes and with the insertion sequence IS*CR1* upstream of *bla* but lacking IS*3000* downstream (GenBank accession no JX026665; Fig. 1). This novel In*60* variant differs from the previously described In*60* integrons in the gene cassette array and also in the absence of IS*3000* and Tn*402* derivatives sequences downstream of $bla_{CTXM\text{-}9}$. In Europe, the $bla_{CTX\text{-}M\text{-}9}$ gene was identified in a variety of IS*CR1*-bearing class 1 integrons among Enterobacteriaceae [2,4], although in *Salmonella* it was usually linked to a conserved In*60* backbone carrying *dfrA16–aadA2* disseminated among humans and poultry and associated with a particular clone of *S.* Virchow [2,3].

The genetic platform bearing $bla_{CTX\text{-}M\text{-}9}$ was located on a transferable 240 kb IncHI2 plasmid carrying a *rep* gene with 99% identity with that of the prototype R478 plasmid (GenBank accession no. NC005211). The spread of CTX-M-9-producing bacteria both of human and animal origin has also been linked to highly related R478-IncHI2 multidrug-resistant (antibiotic/biocides) plasmids [3,4]. As in the case of R478, *arsB* (arsenic) and *terF* (tellurium) genes were detected, but not *merA* (mercury), *silA* (silver/copper) and *copD* (copper) resistance genes. The variability detected in the plasmid content and In*60* platform compared with those previously described suggests the occurrence of multiple recombination events as reported previously [3,4]. The concomitant presence of genes coding for resistance to antibiotics and metals found in the animal setting could contribute to the successful spread of the IncHI2 plasmid family by different events of co-selection. In fact, these multidrug-resistant plasmids are the fifth most common plasmid family occurring in Enterobacteriaceae and have been associated with the dissemination of diverse clinically relevant genes, hence facilitating their increasing prevalence and persistence in different ecological niches.

This study corresponds to the first description of an ESBL-producing *Salmonella* strain in Portugal and the first description of a CTX-M-9 enzyme in the emergent serotype, *S.* Bovismorbificans. The emergence of $bla_{CTX\text{-}M\text{-}9}$ in a similar genetic environment (In*60* variant, IncHI2 plasmid) to those circulating in other *Salmonella* serotypes and *Escherichia coli* from humans and animals in Europe suggests an ongoing spread and evolution of this plasmid in different/new hosts and probably under different selective pressures, requiring surveillance to contain its further dissemination.

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Patrícia Antunes[a,b,*]

[a] *Faculdade de Ciências da Nutrição e Alimentação, Universidade do Porto, Rua Dr Roberto Frias, 4200 Porto, Portugal*

[b] *REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira no 228, 4050-313 Porto, Portugal*

Joana Mourão
Tatiana Alves
Joana Campos
Carla Novais
Ângela Novais
Luísa Peixe

REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira no 228, 4050-313 Porto, Portugal

* Corresponding author. Tel.: +351 22 507 4320; fax: +351 22 507 4329.
E-mail addresses: patriciaantunes@fcna.up.pt, patantunes@gmail.com (P. Antunes)

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First report of bla_{PER-3} in *Acinetobacter baumannii*

Sir,

Acinetobacter baumannii is an important opportunistic nosocomial pathogen with a remarkable ability to survive and disseminate. In addition, *A. baumannii* can acquire new antibiotic resistance genes and upregulate them, making it a problematic pathogen causing numerous outbreaks worldwide [1,2]. Patients at high risk for developing *A. baumannii* infections are those with prolonged hospitalisation and immune suppression, such as cancer patients [2]. Antibiotic selective pressure also contributes to the rising incidence of resistance [1].

The mechanisms of resistance in *A. baumannii* that cause most concern are the β-lactamases, and all the Ambler class enzymes (A–D) have been found in this species. Class D and class B β-lactamases mainly confer resistance to the carbapenems, whilst class A are mainly extended-spectrum β-lactamases (ESBLs) [1]. The first report of an ESBL in *A. baumannii* was bla_{PER-1} in France [3], with PER-like variants in *A. baumannii* now increasingly reported worldwide. PER-3 was initially characterised from an *Aeromonas punctata* in France (GenBank accession no. AY740681.1) and recently in a medical centre in Taiwan [3], sharing 99% homology to bla_{PER-1}. Here we report the first identification of a bla_{PER-3}-producing *A. baumannii* isolate.

Isolate AB-15094 was obtained from a 9-year-old patient with osteosarcoma in 2010 at the Children's Cancer Hospital 57357 (Cairo, Egypt). Phenotypic identification was followed by sequencing of the $bla_{OXA-51-like}$ gene and restriction analysis of the 16S–23S rRNA spacer sequence [2,4].

Minimum inhibitory concentrations (MICs) were determined and interpreted according to British Society for Antimicrobial Chemotherapy (BSAC) guidelines for imipenem, meropenem, ceftazidime and rifampicin. Detection of the OXA-type carbapenemases bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-51} and bla_{OXA-58} as well as class A β-lactamase genes $bla_{PER-like}$, bla_{VEB}, bla_{GES} and bla_{TEM} and the class C β-lactamase gene bla_{ADC} was done by standard PCR [3,4]. The class 1 integron harbouring the bla_{PER-3} gene was analysed by standard PCR and sequencing methods [4]. *S1* nuclease digestion was performed to detect and size plasmids [3]. To investigate whether bla_{PER-3} was plasmid-borne, a plasmid curing experiment was conducted. Susceptibility to ceftazidime was examined after curing and was compared with the initial susceptibility of the uncured strain.

Isolate AB-15094 was confirmed as *A. baumannii* harbouring the bla_{OXA-65} gene. It was sensitive to imipenem and meropenem but resistant to ceftazidime and rifampicin, with MICs of 64 mg/L and 256 mg/L respectively. The isolate was positive for bla_{PER-3} as revealed by sequencing. The strain was negative for all other ESBLs tested as well as for the acquired class D carbapenemases. No insertion sequence (IS) element was detected upstream of the bla_{ADC} gene, suggesting no overexpression of the gene [4].

There was no hybridisation of bla_{PER-3}-specific probes with any of the isolate's three plasmids (60, 70 and 145.5 kb, respectively), suggesting chromosomal localisation of the gene. This was supported by the lack of change in ceftazidime susceptibility after 10 days of plasmid curing. Furthermore, attempted conjugal transfer was also negative. Sequencing upstream of bla_{PER-3} revealed a complex class 1 integron structure harbouring *sul1*, *qacE*Δ1 in the variable 3'CS and ORF*513* directly upstream of the bla_{PER-3} gene, serving as the transcriptional promoter. Fig. 1 shows the schematic representation of the genetic environment of bla_{PER-3}.

This isolate was obtained from a paediatric cancer patient in Egypt and is the first identification of bla_{PER-3} in a clinical *A. baumannii* isolate. Previous reports of this gene have been from *A. punctata* in France and Taiwan where the bla_{PER-3} gene was reported to be localised both on plasmids and on the chromosome [3]; however, in this study it was located on the chromosome alone. The genetic environment upstream of bla_{PER-3} was similar to the *A. punctata* isolate identified in France (GenBank accession no. AY740681.1), being encompassed in a complex class 1 integron. The upstream regions of bla_{PER-3} and bla_{PER-1} differ significantly despite their structural homology. bla_{PER-1} was part of transposon-related structure Tn*1213* consisting of IS*Pa12* and IS*Pa13* flanking the gene [1]. The bla_{PER-3} in this study lacks these IS elements and harbours IS*CR1*, which is closely associated with class A ESBLs and facilitates the movement of these resistance genes [3,5].

PER-3 is a point-mutant derivative of PER-1 [3] harbouring the change Histidine134Leucine. This mutation is positioned in the H4 helix of the secondary structure of the Ω-loop region, which is associated with the active site of class A β-lactamases [5]. The observed mutation could explain the reduced resistance to ceftazidime (MIC = 64 mg/L) in isolate AB-15094 harbouring bla_{PER-3}

Clinical *Salmonella* Typhimurium ST34 with metal tolerance genes and an IncHI2 plasmid carrying *oqxAB-aac(6')-Ib-cr* from Europe

Joana Campos[1]†, Joana Mourão[1]†, Sara Marçal[2], Jorge Machado[3], Carla Novais[1], Luísa Peixe[1] and Patrícia Antunes[1,2]*

[1] UCIBIO/REQUIMTE, Departamento de Ciências Biológicas, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal; [2] Faculdade de Ciências da Nutrição e Alimentação, Universidade do Porto, Porto, Portugal; [3] Laboratório Nacional de Referência de Infeções Gastrointestinais, Departamento de Doenças Infeciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisboa, Portugal.

†These authors contributed equally to the experimental work.

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Clinical *Salmonella* Typhimurium ST34 with metal tolerance genes and an IncHI2 plasmid carrying *oqxAB-aac(6′)-Ib-cr* from Europe

Joana Campos[1]†, Joana Mourão[1]†, Sara Marçal[2], Jorge Machado[3], Carla Novais[1], Luísa Peixe[1] and Patrícia Antunes[1,2]*

[1]*UCIBIO/REQUIMTE, Departamento de Ciências Biológicas, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira no. 228, 4050-313 Porto, Portugal;* [2]*Faculdade de Ciências da Nutrição e Alimentação, Universidade do Porto, Rua Dr Roberto Frias, 4200-465 Porto, Portugal;* [3]*Laboratório Nacional de Referência de Infeções Gastrintestinais, Departamento de Doenças Infecciosas, Instituto Nacional de Saúde Dr Ricardo Jorge, Avenida Padre Cruz, 1649-016 Lisboa, Portugal*

*Corresponding author. Tel: +351-22-5074320; Fax: +351-22-5074329; E-mail: patriciaantunes@fcna.up.pt
†These authors contributed equally to the experimental work.

Sir,

Fluoroquinolones are critical antibiotics for treating severe *Salmonella* infections, and the widespread of resistant isolates included in diverse epidemiological scenarios and carrying plasmid-mediated quinolone resistance (PMQR) is a global threat.[1,2] Among PMQR mechanisms, those encoded by *oqxAB* and *aac(6′)-Ib-cr* genes are of special concern as they also confer reduced susceptibility to other antibiotics (*oqxAB*: chloramphenicol, trimethoprim, olaquindox; *aac(6′)-Ib-cr*: aminoglycosides) and biocides [*oqxAB*: quaternary ammonium compounds (QACs)].[2,3] Although *oqxAB*± *aac(6′)-Ib-cr* are prevalent and widespread in Asia, where olaquindox is still widely used in animal production, they remain scarce in Europe.[1,2,4–6] Here we describe the molecular characterization of clinical ciprofloxacin-resistant *Salmonella enterica* Typhimurium with concomitant presence of *oqxAB* and *aac(6′)-Ib-cr* recovered for the first time in Europe.

Two ciprofloxacin-resistant (MIC 2 mg/L) *S. enterica* Typhimurium isolates carrying *oqxAB* and *aac(6′)-Ib-cr* genes were isolated from faeces of two young children (1 and 2 years old) admitted to a Portuguese hospital in September 2012. Further characterization included the study of susceptibility to 11 other antimicrobial agents and β-lactamase and carbapenemase production by disc diffusion, Etest and/or microdilution methods (EUCAST/CLSI guidelines) and the Blue-Carba test. Screening of genes encoding resistance to antibiotics (including quinolone resistance-determining region mutations and PMQR) or tolerance to biocides/metals found in the animal setting (e.g. feed/disinfectants) was performed by PCR and/or sequencing.[7–9] Determination of transferability of PMQR genes (conjugation at 25/30/37°C and transformation) and characterization of integrons and plasmid backbones were performed by PCR, plasmid-based replicon typing, plasmid MLST (pMLST; http://pubmlst.org/plasmid/), hybridization (I-CeuI/S1-PFGE nuclease) and/or sequencing.[1,7,8] The *oqxAB-aac(6′)-Ib-cr* genetic environment was characterized by PCR mapping based on known sequences.[1] Clonal analysis was assessed by XbaI-PFGE[8] and MLST (http://mlst.warwick.ac.uk/mlst/dbs/Senterica).

The two *Salmonella* Typhimurium isolates were assigned to the widespread ST34 (http://mlst.warwick.ac.uk/mlst/dbs/Senterica) and presented indistinguishable PFGE patterns (Figure S1, available as Supplementary data at *JAC* Online). Clonal expansion of *Salmonella* Typhimurium, including the ST34 clone, with PFGE profiles closely related to those of our isolates, has been associated with the spread of *oqxAB-aac(6′)-Ib-cr* genes in Asian human clinical and food-producing animal isolates.[1,6] However, the isolates reported here could not be related to any outbreak of foodborne infection or to particular food products/animal contact and foreign travel. Additionally, a single *gyrA* mutation (D87N) found in our *Salmonella* Typhimurium strain was identical to that detected in most *oqxAB*-positive *Salmonella* Typhimurium isolates from Asia.[1,6] The strain was co-resistant to amoxicillin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline and trimethoprim (Table 1), and carried diverse metal tolerance genes (*merA*, mercury; *silA*, silver/copper; *pcoD*, copper; *terF*, tellurite). Transferability of *oqxAB-aac(6′)-Ib-cr* was achieved by transformation, with an 8-fold increase in ciprofloxacin MIC and acquisition of resistance to other antibiotics (amoxicillin, chloramphenicol, gentamicin, kanamycin, sulfamethoxazole and trimethoprim) (Table 1). A 2-fold increase in benzalkonium chloride MIC was observed, highlighting the role of the OqxAB efflux pump in the decreasing susceptibility to diverse compounds with antimicrobial activity, including QACs.[3] The concomitant presence of genes encoding resistance to antibiotics (e.g. quinolones, quinoxalines, florfenicol) and biocides/metals (e.g. QACs, copper) widely used in farm animals could contribute to the successful spread of *Salmonella* carrying *oqxAB-aac(6′)-Ib-cr* genes by different events of co-selection.

The *oqxAB* genes were linked to an incomplete class 1 integron containing the *aac(6′)-Ib-cr-bla*$_{OXA-1}$*-catB3-arr3-qacEΔ1-sul1* gene cassette array and flanked by two IS*26* (Figure 1), widely spread mobilizing elements responsible for the dissemination of *oqxAB*[9] and other clinically relevant antibiotic resistance genes.[2] The *oqxAB* genetic environment was identical to that found in Asian *Salmonella* Typhimurium isolates from food-producing animals.[1] In other bacteria (including different *Salmonella* serotypes) similar genetic platforms were observed, although differing in the linkage of *oqxAB* with *bla*$_{CTX-M}$ alleles instead of the gene cassette array.[2,4,5] This IS*26-oqxAB*-IS*26-aac(6′)-Ib-cr-bla*$_{OXA-1}$*-catB3-arr3-qacEΔ1-sul1*-IS*26* was located on a 180 kb IncHI2 plasmid carrying a *rep* gene 100% identical to Asian *Salmonella* Typhimurium pHXY0908 bearing *oqxAB-aac(6′)-Ib-cr* genes (GenBank accession number KM877269), but with only 95% identity to that of the prototype R478 (GenBank accession number BX664015) and prototype pAPEC-01-R (GenBank accession number DQ517526)

Research letter

Table 1. Antimicrobial susceptibility phenotypes and genotypes of *Salmonella* Typhimurium isolates (234 and 248), the selected transformant (248_T) and the recipient (*Escherichia coli* DH5α)

	MIC (mg/L)				
Isolate	CIP[a]	NAL[b]	BZK	Resistance phenotype[c]/genotype to other antimicrobial agents	Metal tolerance genes
234	2	>256	32	AMX, CHL, GEN, KAN, STR, SUL, TET, TMP/bla_{OXA-1}-bla_{TEM}, *catB3-cmlA-floR*, *aac(3)-IV*, *aphA1*, *aadA-strA-strB*, *sul1-sul2-sul3*, *tet*(B), *dfrA12*	*merA*, *silA*, *pcoD*, *terF*
248	2	>256	32	AMX, CHL, GEN, KAN, STR, SUL, TET, TMP/bla_{OXA-1}-bla_{TEM}, *catB3-cmlA-floR*, *aac(3)-IV*, *aphA1*, *aadA-strA-strB*, *sul1-sul2-sul3*, *tet*(B), *dfrA12*	*merA*, *silA*, *pcoD*, *terF*
248_T	0.25	128	16	AMX, CHL, GEN, KAN, SUL, TMP/bla_{OXA-1}, *catB3-cmlA-floR*, *aac(3)-IV*, *aphA1*, *aadA*, *sul1-sul2-sul3*, *dfrA12*	*terF*
E. coli DH5α	0.03	64	8		

[a]PMQR genes screened: *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA*, *aac(6′)-Ib-cr* and *oqxAB*.
[b]*Salmonella* isolates presented a single *gyrA* mutation D87N.
[c]Susceptibility to amoxicillin (AMX), benzalkonium chloride (BZK), ciprofloxacin (CIP), chloramphenicol (CHL), gentamicin (GEN), imipenem, kanamycin (KAN), nalidixic acid (NAL), streptomycin (STR), sulfamethoxazole (SUL), tetracycline (TET) and trimethoprim (TMP) was determined.

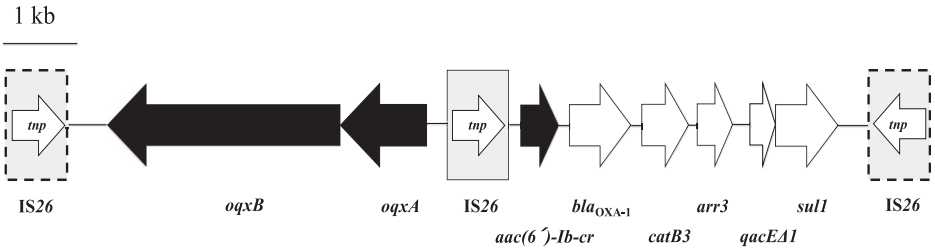

Figure 1. Genetic environment (12 110 bp) of *oqxAB-aac(6′)-Ib-cr* genes in an IncHI2 plasmid from an ST34 *Salmonella* Typhimurium strain. Horizontal arrows represent the positions and transcriptional directions of the ORFs. The IS*26* elements are shown as light grey boxes and dotted outlines indicate incomplete sequences.

plasmids. Also, among the metal tolerance genes found, our IncHI2 plasmid and pHXY0908 carried only *terF*, contrasting with the prototypes R478 and pAPEC-01-R with multiple metal tolerance genes (e.g. *merA*, *arsB*, *silA* or *pcoD*). The spread of *oqxAB*±*aac(6′)-Ib-cr*-producing bacteria of both human and animal origin in Asia has been linked mostly to IncHI2 MDR plasmids[1,4–6] belonging to ST1/ST2 by pMLST,[4] the two major plasmid variants associated with the dissemination of diverse clinically relevant genes (e.g. bla_{ESBL}).[10] Our MDR IncHI2 plasmid was non-typeable by pMLST (lacking allele smr0199/smr0018=allele number 3), suggesting the occurrence of multiple recombination events and of a new variant, considering those circulating in Europe (http://pubmlst.org/plasmid/).[10]

To the best of our knowledge, we describe for the first time in Europe an MDR *Salmonella* strain harbouring both *oqxAB* and *aac(6′)-Ib-cr* PMQR genes within an IncHI2 plasmid. The spread of these bacteria and/or MDR plasmids, as is occurring in Asia in human/animal settings, is of concern since they may contribute to amplification of *oqxAB-aac(6′)-Ib-cr* among Enterobacteriaceae in Europe, probably under different selective pressures (e.g. antibiotics/biocides/metals). Continuous surveillance to contain further transmission of these PMQR genes for new hosts or different settings, facilitated by international human and animal/food travel, is urgently required to preserve critical compounds with antimicrobial activity, including fluoroquinolones.

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Transparency declarations

None to declare.

Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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Figure S1 is available as Supplementary data at *JAC* online

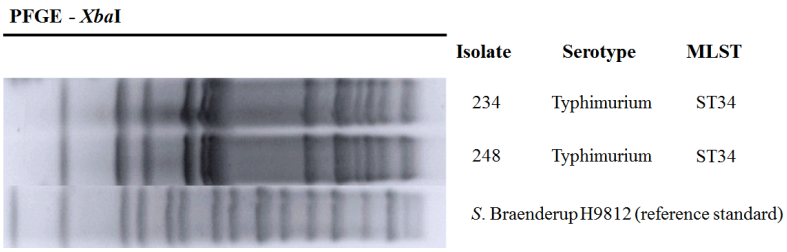
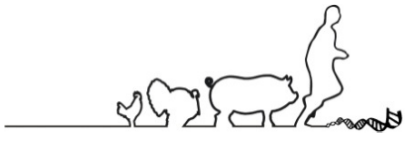


Figure S1. PFGE patterns of clinical ciprofloxacin-resistant *S. enterica* isolates carrying *oqxAB* and *aac-6'-Ib-cr* genes recovered from two young children admitted in a Portuguese hospital (2012). The *Salmonella* serotype and multilocus sequence type (MLST) are also indicated.



Chapter 4

Conclusions

"Anyone who has never made a mistake has never tried anything new."

Albert Einstein

Overall, the results of this thesis conducted to the following **general CONCLUSION**:

The acquisition of metal tolerance genes, particularly to copper and silver, confers an advantage to particular clinically relevant multidrug-resistant *Salmonella* serotypes/clones by facilitating their better survival and persistence in environments contaminated with those metals across the food chain.

Further conclusions are summarized as follows:

1) Pig production setting is an important source of clinically relevant *Salmonella* serotypes/clones

- Clinically relevant MDR *Salmonella* serotypes/clones are particularly associated with pig setting in Portugal (2002-2014), namely to piggeries environment and pork products. These MDR *Salmonella* serotypes/clones were mainly identified as *S.* Typhimurium (European clone), *S.* 4,[5],12:i:- (European, Spanish and Southern European clones) and *S.* Rissen/ST469. They are a public health issue emergent in diverse European regions and now well characterized (e.g. clonality, MDR profiles and associated genetic platforms, sources) in our country.

- The presence of typical molecular patterns in clones of the worldwide epidemic *S.* 4,[5],12:i:- as well as *S.* Typhimurium European clone and *S.* Rissen highlights the ability of those serotypes/clones to acquire and maintain different adaptive features (e.g. MDR) with potential impact in their emergence. This was demonstrated for the most prevalent *S.* 4,[5],12:i:- European (ASSuT phenotype, RR1-RR2 resistance regions, ST34) and Spanish clones (ACGSSuTTm phenotype, *sul3*-type III integron within IncA/C plasmids, ST34) but also for the less frequent Southern European clone (CSSuTTm phenotype, *sul3*-type I integron within IncR plasmids, ST19), both in isolates from human clinical samples, food, environment and piggeries. Also, *S.* Rissen (ASSuTTm phenotype, ST469) and *S.* Typhimurium European clone (ASSuT phenotype, ST34) carried particular features.

2) Epidemiology of metal tolerance in *Salmonella*

Acquired metal tolerance genes were dispersed in diverse *Salmonella* genetic backgrounds, especially in clinically relevant MDR pig-associated serotypes or clones

- Genes coding for copper (*pcoA-pcoD*/*silA-silE*), silver (*silA-silE*) and mercury (*merA*) tolerance were detected in isolates from diverse sources, clones and serotypes and were more frequently observed than those coding for arsenic (*arsB*) or tellurite (*terF*) tolerance, only found in few isolates/serotypes. The dispersion of those genes in *Salmonella* from different serotypes and clones suggests a genetic exchange between members sharing the same ecological communities and hosts potentially subjected to similar metal environmental stresses.

- *Salmonella* of emerging pig-associated serotypes/clones are more enriched in genes coding for copper and silver (*silA-silE±pcoA-pcoD*) than *Salmonella* serotypes/clones associated with other food-animal settings, which is possibly explained by variable selection contexts of animal production practices. This is supported by the allowed use of higher copper amounts in feed for pigs comparing with other animals, which might have contributed for the selection and persistence of emerging *Salmonella sil±pco*$^{+}$, particularly the MDR serotypes/clones *S.* 4,[5],12:i:- European clone/ST34, *S.* Typhimurium European clone/ST34 and *S.* Rissen/ST469. In contrast, *sil* and *pco* genes were absent in several poultry/eggs-associated serotypes (e.g. *S.* Enteritidis, *S.* Infantis, *S.* Heidelberg), a setting where only low Cu concentrations are allowed in Europe. Nevertheless, an often occurrence of *sil±pco* in available genomes of emerging poultry-associated serotypes (e.g. Heidelberg, Kentucky), suggests that different production practices (e.g. in USA copper supplements are not restricted by animal type) might contribute for the selection and emergence of different *Salmonella* serotypes or strains.

- *Salmonella* isolates carrying *sil±pco* genes coding for copper/silver tolerance were more resistant to antibiotics than those without these genes clusters. This data highlights the importance of carrying different genetic features involved in an improved survival capacity to environments subject to different stressors (e.g. metals or antibiotics), which might acount for the expansion of MDR *Salmonella* serotypes/clones across the food-animal setting.

- The combination of *sil±pco* genes can be used as additional molecular biomarkers of current emerging clones in Europe. This is supported by the occurrence of the *sil+pco* genes in almost all *S.* Rissen, *S.* 4,[5],12:i:- and *S.* Typhimurium European clones and the *sil* gene alone in *S.* 4,[5],12:i:- Spanish and Southern European clones.

3) Metal tolerance and antibiotic resistance: characterization of genetic elements and horizontal gene transfer

Variable genetic platforms contribute for the co-dispersion of metal tolerance and antibiotic resistance genes

- Metal tolerance and antibiotic resistance genes are often co-located in the same genetic platforms, sharing either the same plasmid or chromosomal regions in diverse *Salmonella* serotypes. Diverse metal tolerance genes were located in the chromosome (*sil*±*pco*, *merA, terF*) or in large plasmids (*sil*±*pco*, *merA*, *terF*, *arsB*) along with those coding for resistance to other antimicrobial agents as biocides (e.g. *oqxAB*-quaternary ammonium compounds) or antibiotics (e.g. ampicillin-$bla_{TEM,}$ tetracycline-*tet* or sulphonamides-*sul* genes) including some relevant in the treatment of *Salmonella* infections (e.g. $bla_{CTX\text{-}M\text{-}9}$-extended-spectrum cephalosporins, or *aac(6')-Ib-cr*/*oqxAB*-fluoroquinolones). Also, the combination of particular metal (*sil*±*pco* and/or *merA*) and antibiotic resistance (e.g. *tet* genes, typical-*sul1* and/or atypical *sul3*-type class 1 integrons) genes found within these genetic platforms might also reflect their abundance in the animal setting and thus the probability to recombine and accumulate in the same genetic platforms.

- Large plasmids carrying metal tolerance genes belong to families widely identified in *Enterobacteriaceae* and often involved in antibiotic resistance spread. This is supported by the detection of metal tolerance genes (*sil*±*pco*, *merA*, *terF, arsB*) in major plasmid families such as IncA/C, IncR, IncFIIA, IncHI1, IncHI2, IncN, IncP and IncI1, some of them transferable. Although some of those plasmids (e.g. IncHI2) have been frequently associated with diverse metal tolerance genes, we unveiled the relevance of IncFIIA and IncN plasmids in the dispersion of such genes. These data also alerts for the importance of single horizontal transfer events in the co-dispersion of both metal tolerance and clinically relevant antibiotic resistance genes [e.g. *aac(6')-Ib-cr*/*oqxAB*, $bla_{CTX\text{-}M\text{-}9}$] among bacteria from different species sharing the same ecosystems and subjected to diverse selective pressures.

- Chromosomal location of the *sil*±*pco* genes coding for copper/silver tolerance and/or *merA* were mainly observed in the MDR European clone of *S.* Typhimurium and *S.* 4,[5],12:i:- and in the *S.* Rissen. These data along with the inability of transfer chromosomal genetic platforms in conjugation assays highlight the importance of clonal spread for the maintenance of *sil*±*pco* in *Salmonella* recovered from the food-animal setting. However, the need of critical and particular conditions for the mobilization of

chromosomal genetic platforms carrying *sil±pco* in laboratory assays could not be discarded.

- The *sil* and *pco* gene clusters are adjacent in the same genetic region and are inserted in a Tn*7*-like element, the Tn*6230*, both in plasmids and the chromosome, particularly in recently emergent *Salmonella* serotypes/clones. This was supported by our data and/or by a detailed *in silico* genomic analysis of available *Salmonella* genomes from different sources and serotypes. In some cases, the absence of transposition proteins of Tn*6230*-like elements could be related to recombination events potentially associated with the fixation of these elements in diverse *Salmonella* serotypes. Further studies are needed to clarify the contribution of Tn*6230*-like elements in the emergence of particular *Salmonella* serotypes/clones as *S.* Typhimurium and *S.* 4,[5],12:i:-.

4) Copper and silver tolerance phenotypes in *Salmonella*

Higher tolerance to $CuSO_4$ in anaerobic atmosphere and to $AgNO_3$ after prior exposure to silver was observed among the *sil*+*pco*$^+$ or *sil*$^+$ isolates

- Wild type *Salmonella* isolates and transconjugants carrying the *sil* efflux system, independently of the chromosomal or plasmid location, were associated with higher tolerance to $CuSO_4$ in anaerobiosis and to $AgNO_3$ after prior exposure to silver in aerobiosis. These data contributed to demonstrate the role of the *sil* gene cluster in *Salmonella* adaption to metal contaminated environments, namely to overcome the more toxic Cu^+ occurring in reduced settings potentially present in food-animal productions (e.g. animal gut of livestock carriers, manure, waste lagoons or forage/feed, sewage and sludge).

- Further studies are needed to understand the "biocidome" associated with non-typhoidal *Salmonella*: i) to clarify the potential role of the *pco* gene cluster in other important adaptive features (e.g. virulence); ii) to unveil other genetic determinants associated with copper tolerance, which could explain the presence of high levels of aerobic $CuSO_4$ tolerance in *Salmonella* isolates, carrying or not *pco* genes, or the occurrence of high anaerobic $CuSO_4$ tolerance in isolates lacking the known *sil±pco* gene clusters.

Modified methodological approaches in Cu/Ag tolerance assays are critical to identify isolates carrying *sil*±*pco* gene clusters

- The use of anaerobiosis to determine *Salmonella* $CuSO_4$ tolerance and the prior contact with subinhibitory concentrations of $AgNO_3$ is mandatory for the detection of the higher MICs to these compounds determined by *sil*±pco acquisition, independently of the *Salmonella* serotype/clone. The inclusion of anaerobiosis, here firstly applied to the standard method using aerobiosis, was considered because *silE* binds to the more toxic form Cu^+ in anaerobiosis. In the case of $AgNO_3$, despite of *sil* gene cluster had been reported as being inducible in the presence of silver, few studies used this approach (applied a prior contact with silver before MIC determination) or the methodological details were poorly described, impairing its reproducibility. Thus, well-described protocols allowing reproducible results are published and available in this thesis (Chapter 5 - Appendix) for general use.

- Here we have proposed tolerance cut-offs to $CuSO_4$/$AgNO_3$ that can be used in further studies in order to distinguish *Salmonella* isolates with and without *sil* genes, which will have a significant impact in data comparison from different studies. This is critical, as currently the use of different methodologies is associated with an unclear link between phenotypic and genotypic data related with *sil* acquisition, which hinders the understanding of copper and silver tolerance in *Salmonella*. The application of these cut-offs is only suitable when the modified methodological approaches proposed are applied.

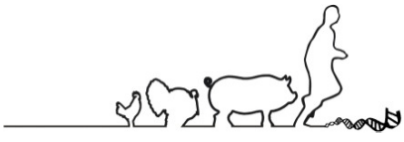
CLOSING REMARKS

Different gaps in the field of metal and biocide tolerance knowledge have been pointed out by different entities such as the European Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR). The lack of information on this topic has hindered the understanding of the impact of the use of biocides and metals in public health. Among other issues, it is referred the lack of monitoring programs, which limits the gathering of robust epidemiological data and knowledge about the selection and spread of antibiotic resistance genes associated with the use of metals and biocides. It is also denoted the absence of standardized methods for the evaluation of bacterial susceptibility to these compounds.

In *Salmonella*, a foodborne zoonotic pathogen of clinical importance with ability to genetically interact with different microorganisms in diverse biological communities, the results of this study contributed to:

a) Know the epidemiology of tolerance to copper, silver, mercury, arsenic and tellurium using, for the first time, a large collection of *Salmonella* isolates (n=406) representative of different epidemiological and genetic backgrounds (niches/sources, time frames, serotypes and clones). It was showed that the pig production setting seems to be a relevant reservoir of successful and worldwide emerging MDR *Salmonella* serotypes/clones enriched in different adaptive features (besides antibiotic resistance or virulence genes), including tolerance to metals currently used in food-animal setting.
b) Understand the potential role of metals, particularly copper and silver, widely present in different anthropogenic contexts, in the selection and maintenance of antibiotic-resistant *Salmonella*, through the characterization of the genetic elements harbouring metal tolerance and antibiotic resistance genes.
c) Develop more efficient methodological approaches for detecting copper and silver tolerance and to establish tolerance cut-offs to $CuSO_4$/$AgNO_3$, which will allow the comparision of data in further studies.

The new information provided by this study might help to support the implementation of more efficient intervening measures related to the use and/or accumulation of metals in diverse environments, in order to prevent a wider expansion of foodborne zoonotic pathogens such as MDR *Salmonella* serotypes/clones or the emergence of new ones.



Appendix

"If you want to live a happy life, tie it to a goal, not to people or objects."

Albert Einstein

I. Review paper – "Salmonellosis: the role of poultry meat"

Salmonellosis: the role of poultry meat

CMI Themed Issue "Poultry and human infections"

Patrícia Antunes[1, 2], Joana Mourão[2], Joana Campos[2] and Luísa Peixe[2]*

[1] Faculdade de Ciências da Nutrição e Alimentação, Universidade do Porto, Rua Dr. Roberto Frias, 4200 Porto, Portugal; [2] UCIBIO/REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, no. 164, 4050-047 Porto, Portugal.

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REVIEW

Salmonellosis: the role of poultry meat

P. Antunes[1,2], J. Mourão[2], J. Campos[2] and L. Peixe[2]

1) *Faculdade de Ciências da Nutrição e Alimentação, Universidade do Porto and* 2) *UCIBIO/REQUIMTE. Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal*

Abstract

Salmonellosis remains one of the most frequent food-borne zoonoses, constituting a worldwide major public health concern. Currently, at a global level, the main sources of infection for humans include meat products, including the consumption of contaminated poultry meat, in spite of the success of *Salmonella* control measures implemented in food-animal production of industrialized countries. In recent years, a shift in *Salmonella* serotypes related to poultry and poultry production has been reported in diverse geographical regions, being particularly associated with the spread of certain well-adapted clones. Moreover, antimicrobial resistance in non-typhoidal *Salmonella* is considered one of the major public health threats related with food-animal production, including the poultry production chain and poultry meat, which is an additional concern in the management of salmonellosis. The circulation of the same multidrug-resistant *Salmonella* clones and/or identical mobile genetic elements encoding antibiotic resistance genes from poultry to humans highlights this scenario. The purpose of this review was to provide an overview of the role of poultry meat on salmonellosis at a global scale and the main problems that could hinder the success of *Salmonella* control measures at animal production level. With the increasing globalization of foodstuffs like poultry meat, new problems and challenges might arise regarding salmonellosis control, making new integrated intervention strategies necessary along the food chain.

Keywords: AmpC, extended-spectrum β-lactamases, multidrug-resistance, plasmid-mediated quinolone resistance, *Salmonella*, Enteritidis, Infantis, Kentucky, Heidelberg, Typhimurium, Virchow

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Corresponding author: L. Peixe, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira nº 228, 4050-313 Porto, Portugal
E-mail: lpeixe@ff.up.pt

Introduction

Salmonella infections are a worldwide major public health concern; Salmonellosis is caused by non-typhoidal *Salmonella enterica* serotypes (serotypes other than S. Typhi and S. Paratyphi) and is typically characterized by a self-limiting gastroenteritis syndrome (manifested as diarrhoea, fever and abdominal pain), with an incubation period between 4 and 72 h and mortality being rare [1–3]. In healthy humans, the infectious dose is generally 10^6 to 10^8, but lower bacterial counts can cause disease in certain conditions, as well as in infants and the elderly [2]. Although uncommon, life-threatening invasive infections with bacteraemia (5%–10% of infected persons) and/or other extra-intestinal infections may occur, affecting especially the risk groups (infants, young children, older people and immunocompromised patients) [1–3]. In severe cases, effective antimicrobial agents are essential, so the emergence of *Salmonella* that are resistant to critical antibiotics is of concern [1,2].

In industrialized countries, the main reservoir of non-typhoidal *Salmonella* is the intestinal tract of food-producing animals, which readily leads to contamination of diverse foodstuffs [3–5]. Therefore, despite other sources (e.g. contact with animals/reptiles, environment or person-to-person), food-borne salmonellosis is the most relevant source with a high

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global impact in human health. It was estimated that non-typhoidal *Salmonella* causes around 93.8 million illnesses and 155 000 deaths each year worldwide [6]. In the USA, more than 1 million annual cases of food-borne salmonellosis were estimated by the CDC; they were associated with the largest number of hospitalizations and deaths compared with other food-borne microbial agents [7]. In Europe, salmonellosis has been the second most common zoonosis (82 694 confirmed cases and 20.4 cases per 100 000 population in 2013) and the most frequent cause of food-borne outbreaks, in spite the reported decreasing trend that has resulted from *Salmonella* control programmes [8].

Although different serotypes have been associated with salmonellosis, a limited number are responsible for most human infections; *S. enterica* Enteritidis being the most frequent one in the EU (39.5% in 2013) and USA (14.5% in 2012) followed by *S. enterica* Typhimurium (including its monophasic variant) (28.8% in EU, 2013; 11.6% in USA) [8,9]. *Salmonella* Enteritidis is commonly associated with poultry and products thereof, whereas *S.* Typhimurium has a wider species range, including pigs and cattle as well as poultry [10]. Therefore, foods of animal origin, in particular contaminated poultry products (eggs and poultry meat) have been considered the main vehicles of *Salmonella* infection and clearly associated with the worldwide epidemic of *S.* Enteritidis [4,5,11,12]. Moreover, diverse epidemiological studies have supported the great contribution of poultry foodstuffs to the salmonellosis burden [4,5,12,13].

In recent years, with the implementation of *Salmonella* control programmes mainly in poultry production (e.g. in the EU and USA), changing trends in food-borne salmonellosis and associated serotypes were observed, with the expansion of previously less common serotypes, frequently resistant to antibiotics. With the increasing globalization of foodstuffs like poultry meat, which is one of the most consumed and increasingly globally traded meat products, new problems might arise regarding salmonellosis control. An overview of the role of poultry meat in salmonellosis at a global scale is provided in this review.

Non-typhoidal *Salmonella* and poultry meat

Poultry populations, in particular chicken and turkey, are frequently colonized with *Salmonella* without detectable symptoms (sub-clinical infections/healthy carriers) by horizontal and vertical transmission at primary production level [4,5]. The presence of *Salmonella* in healthy poultry animals is suggested as the main risk factor, by allowing bacteria to easily transmit in table eggs and poultry meat to humans [10]. In fact, in Europe it is assumed that the observed reduction in salmonellosis cases (32% between 2008 and 2012) is mainly due to successful *Salmonella* control measures (involving surveillance, biosecurity and vaccination) implemented in poultry/egg production and focused on particular serotypes (e.g. *S.* Enteritidis and *S.* Typhimurium) that are considered of public health significance [4,8,10,12]. These measures led to the achievement of reduction targets for poultry populations in most EU countries and lower non-compliance regarding *Salmonella* in poultry products [4,8]. Moreover, decreasing contamination rates in raw poultry products are in agreement with those recently observed in industrialized countries from other geographical regions with pathogen reduction programmes, such as the USA [12,14,15]. It should be noted that by the 2000s a high incidence of *Salmonella* in poultry products was reported in the EU, with rates >50% for several countries [16]. In the 2013 zoonosis EFSA/ECDC report involving data from European countries, as in previous years, *Salmonella* was most frequently reported, although at low levels, in fresh turkey (5.4%) and fresh broiler meat (3.5%), in comparison with eggs (0.1%) or fresh pig meat (0.7%) [8]. Despite the highest incidence being detected in poultry meat, eggs still remain the most important source of food-borne *Salmonella* outbreaks [8]. In fact, using quantitative source attribution models the higher number of human salmonellosis cases in Europe was attributable to eggs (65% in 2011 and 17% in 2012) and pigs (28% in 2011 and 56.8% in 2012) compared with broilers (2.4% in 2011 and 10.6% in 2012) and turkey meat (2.6% in 2011 and 4.5% in 2012) [17,18]. However, diverse surveys targeted to detect *Salmonella* in poultry products in developing countries, some with expansion of the poultry industry, still detected high percentages of positive samples, ranging from ~13% to 39% in South America [19,20], ~35% in Africa [21,22] and ~35% to 50% in Asia [23–25]. Those differences possibly reflect diverse poultry production husbandry practices and absence of control measures along the food chain, highlighting the importance for *Salmonella* spread of the extensive international trade in animals and their products [4].

Worldwide data about *Salmonella* serotype prevalence in humans and in the diverse range of foodstuffs have contributed to establish an epidemiological link between salmonellosis and poultry products, with diverse serotypes overlapping between humans and poultry meat (chicken and turkey) (Fig. 1). In the EU, recent changes in the frequency of *Salmonella* serotypes causing human infections were reported, which in some cases were in line with those occurring in poultry (Fig. 1). Nevertheless, interpretation of these data should be cautious, owing to limitations in the number of poultry isolates serotyped each year. Of particular relevance is the decrease in *S.* Enteritidis human cases (19% reduction between 2011 and 2013 in the EU), a serotype typically associated with poultry meat and egg

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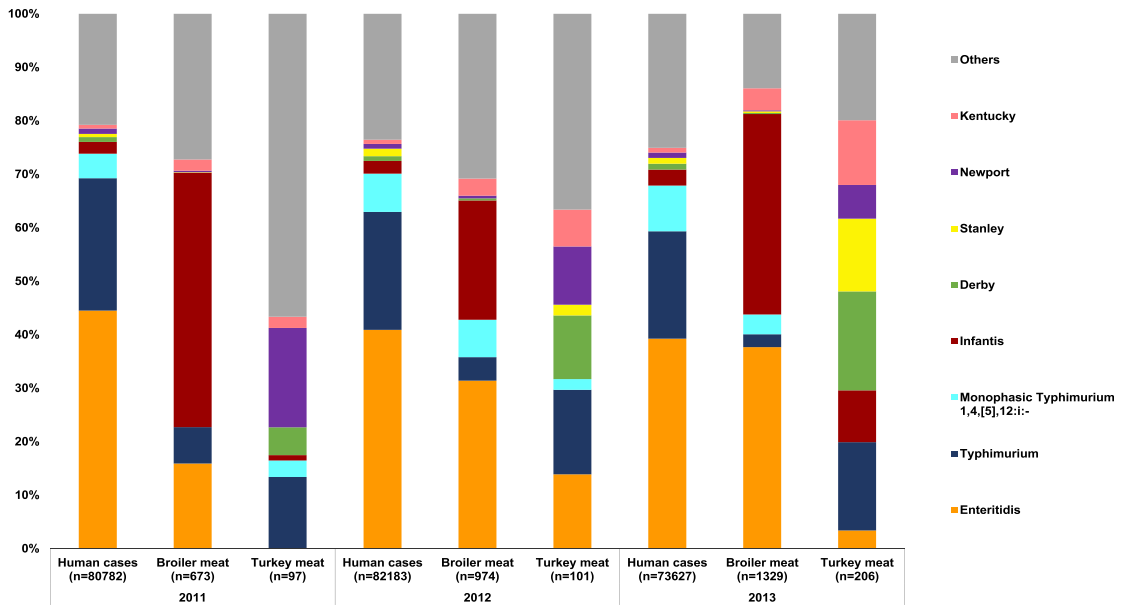

FIG. 1. Distribution of the major serotypes of non-typhoidal *Salmonella* associated with human cases (salmonellosis) and poultry meat in EU, 2011 to 2013. Data were obtained from EFSA reports: humans (2011–2013) and turkey/broiler meat (2013) [8]; turkey/broiler meat (2011–2012) (http://www.efsa.europa.eu/en/efsajournal/pub/3129 and http://www.efsa.europa.eu/en/efsajournal/pub/3547) (last accessed 1 September 2015). The percentages were calculated based on the total number of serotyped isolates per type of meat or human salmonellosis cases.

consumption, in line with a decreasing trend in poultry and poultry products pointed by different studies [8,12,26–29]. Increasing occurrences of other serotypes implicated in human infections (e.g. *S.* Infantis, *S.* Stanley, *S.* Kentucky) related with poultry meat (chicken and turkey) have been reported in the EU (Fig. 1). However, one major difference in the serotype pattern between humans and poultry is related to *S.* Typhimurium and its monophasic variant, both frequently associated with human salmonellosis, but less common in poultry meat, pigs and pig meat being the main source [8]. In USA and Canada, other serotypes, like *S.* Heidelberg and *S.* Kentucky, have emerged as predominant serotypes in poultry and have also been implicated in human salmonellosis, beyond *S.* Enteritidis [9,12,14,15,28,30–32].

The shift in *Salmonella* serotypes related to poultry and poultry production has been associated with the spread of certain clones. For instance, the *S.* Infantis increase (26.5% in human cases between 2011 and 2013 accounting for the fourth most common serotype and the most reported in broilers and broiler meat) [8], has been associated with the spread of several clones of broiler origin in diverse European countries, including the dominant Hungarian clone [33–35]. Also, *S.* Stanley showed an increase since 2011 with a peak in 2012, being the sixth most common human serotype and one of the three most common in turkey meat, together with *S.* Derby and *S.* Kentucky in 2013 [8]. A large *S.* Stanley outbreak caused by a new clone (novel Pulsed Field Gel Electrophoresis-type), was linked with the consumption of turkey meat, and is still circulating in the European food market (at least since 2011), with a considerable risk of becoming endemic in the poultry production chain in Europe [36–38]. In the USA, *S.* Heidelberg in particular has been identified as one of the top human and poultry serotypes, with several clones implicated in diverse large multistate outbreaks resulting from the consumption of contaminated chicken or turkey products [12,31]. The spread and the global persistence of serotype *S.* Kentucky reflect other particular situations related to the increased globalization of travel and the food/animal trade in different geographical regions. This serotype has been associated with a worldwide (Europe, Africa and Asia) spread of a particular epidemic clone (*S.* Kentucky ST198-X1), recovered from several livestock reservoirs, particularly poultry farms, with chicken and turkey implicated as the potential major human infection vehicles [39–43]. These and other examples of multi-country/multi-state outbreaks or clonal expansion of *Salmonella* infections linked to poultry meat (Table 1) serve as a reminder of the importance of acting upon any *Salmonella* contamination in the food chain and monitoring to detect the emergence of any serotype or new clone. This

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TABLE 1. *Salmonella* outbreaks and emerging clones linked with poultry meat products (2002–ongoing)

Serotype (outbreak/clone designation)[a]	Source	Year(s)[b]	Geographical region	No. of cases[c]	Reference
Enteritidis (outbreak)	Chicken	2015–	USA	3	CDC, 2015[d]
Enteritidis (outbreak)	Chicken	2015–	USA	9	CDC, 2015[d]
Hadar (outbreak)	Turkey	2010–2011	USA	12	CDC, 2015[d]
Heidelberg (outbreak)	Chicken	2013–2014	USA, Puerto Rico	634	CDC, 2015[d]
Heidelberg (outbreak)	Chicken	2013	USA	9	CDC, 2015[d]
Heidelberg (outbreak)	Chicken	2012–2013	USA	134	CDC, 2015[d]
Heidelberg (outbreak)	Chicken	2011	USA	190	CDC, 2015[d]
Heidelberg (outbreak)	Turkey	2011	USA	136	CDC, 2015[d]
Infantis (Israel clone)	Broiler chickens	2007–2009	Israel	NA	[26]
Infantis (Hungarian clone)	Broilers and chicken	2004–2009	Europe	NA	[33–35]
Kentucky (ST198-X1)	Chicken and turkey	2002–2013	Europe, Africa, Asia	NA	[39,40,42]
Stanley (outbreak)	Turkey	2011–2013	Europe	710	[36,37]

[a]ST, sequence type.
[b]'year-' include ongoing reported outbreaks.
[c]Estimated number of cases only when outbreaks were reported. NA, not applicable in emerging clones.
[d]CDC, 2015. *Salmonella*. Reports of selected *Salmonella* Outbreak Investigations. Available at: http://www.cdc.gov/salmonella/outbreaks.html [accessed September 2015].

scenario also remains alert for the need for inclusion of those serotypes, or particular clones, considered to be of public health significance, in control and surveillance programmes [10,12,38].

Salmonella serotypes and clones associated with human infections and with an enhanced ability to colonize several food animals, able to persist along the food chain (e.g. primary production on-farm, slaughter operations, equipment and meat handlers, retail meat) with efficient transmission and rapid spread are of public health relevance [11,34,43–45]. Although understanding the exact mechanisms of their persistence and spread in poultry production are still largely unknown, recent studies focusing on emergent poultry-associated *Salmonella* strains unveiled specific features that could provide a significant advantage both in the environment and in the host (poultry/human) [12]. For example, in Israel, an *S.* Infantis emergent clone possessed a megaplasmid, which increased its tolerance to stress factors (e.g. mercury and oxidative stress) and its virulence/pathogenicity (e.g. enhanced biofilm formation, adhesion and invasion into avian and mammalian host cells) [46]. Also, a genomic study of several predominant *Salmonella* serotypes from Canadian broiler chickens showed the presence of multiple features related with pathogenicity (e.g. genes encoding adhesins, flagellar proteins, iron acquisition systems, type III secretion system) and stress tolerance (e.g. metal and antiseptic tolerance genes; better acid-stress response) [32]. S. Heidelberg, including ground turkey outbreak isolates, carried phages and plasmids with diverse virulence factors (e.g. P2-like phage-*sopE1* gene, IncX-type IV secretion system), which could play a role in their virulence (a serotype highly related to invasive infections), colonization and persistence (a poultry-associated serotype) [12,31]. In *S.* Kentucky, the acquisition of an *E. coli* ColV virulence plasmid was also associated with enhanced colonization ability in chicken, particularly in a dominant avian clonal type [12]. These features can play a role in the successful spread of emergent and virulent serotypes/clones that could contribute in a short time to replacing other *Salmonella*. Moreover, those emergent *Salmonella* serotypes are usually enriched with antimicrobial resistance determinants conferring multidrug-resistance [12,31,32,46], which is currently one of the major public health concerns (discussed in the next section).

Antimicrobial-resistant non-typhoidal *Salmonella* and the poultry linkage

The emergence and spread of *Salmonella* isolates presenting resistance to several antibiotics is of concern because these medicines are crucial to the successful treatment of invasive infections [1,11]. Since resistance to older antibiotics (e.g. ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole) has been increasing for many years, recommended treatment options for salmonellosis included fluoroquinolones (ciprofloxacin) and extended-spectrum cephalosporins [1,2]. However, resistance to those 'critically important antibiotics for human health' is emerging, leading to increased severity, morbidity and mortality of diseases and the need for the use of last-line antimicrobials (e.g. carbapenems) in therapy [47].

For several decades, the contribution of the food-animal as a reservoir of antimicrobial resistance with impacts on human health has been controversial, but accumulating evidence linking particularly poultry production with human disease have been reported, namely involving non-typhoidal *Salmonella* [48–50]. The first evidence is the close association between the use of antimicrobial agents and the occurrence of resistance. In fact, regular use of antibiotics (mainly for animal health and in some countries as growth promoters), a practice associated with modern intensive food-animal/poultry production, has been

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considered the main driver for the development of antibiotic resistance in zoonotic bacteria, such as *Salmonella* [48,50,51]. For example, licensing of the fluoroquinolone enrofloxacin for animal use, especially in poultry, in the 1990s led to increased rates of decreased susceptibility to ciprofloxacin in *S.* Typhimurium DT104 recovered from animal/food (particularly poultry) and humans [5]. Other findings points to a link between resistance to nitrofurans in human *Salmonella* and the food chain, suggesting that its illegal use in the poultry industry might have contributed to the selection and persistence of *S.* Enteritidis in poultry, and consequently to human salmonellosis in Portugal [52]. More recently, a voluntary withdrawal of ceftiofur by the poultry producers in Canada was correlated with a decreasing occurrence of ceftiofur-resistant *S.* Heidelberg (one of the most common serotypes associated with salmonellosis in this country) from both human infections and retail poultry, with an increase of the resistance levels after reintroduction of use [30].

Further evidence for the impact of poultry production on human health problems associated with antimicrobial resistance in *Salmonella* is the correlation between different reservoirs (humans and food-animals) obtained from systematic surveillance data. In the last EU report, *Salmonella* resistant (R) or multidrug-resistant (MDR) to commonly used antimicrobials was frequently detected in humans (R = 50%; MDR = 31.8%) and animals, especially broilers (MDR = 56%) and turkeys (MDR = 73%), but also in derived meat products [43]. Also, in the USA, high levels of resistance and MDR in chicken (R = 60%; MDR = 26%) and turkey (R = 77%; MDR = 39.6%) seems to greatly contribute to levels in humans (R = 19%; MDR = 10%) [53]. Those data about MDR are extremely worrying because of the possible role of diverse antibiotics in the co-selection of *Salmonella* strains resistant to clinically relevant antibiotics, such as fluoroquinolones and extended-spectrum cephalosporins. In the EU, relatively low levels of *Salmonella* non-susceptible ('clinically' resistant and 'intermediate' resistant categories combined) to ciprofloxacin (3.8%) and 'microbiological' resistant (non-wild type by epidemiological cut-off values) to cefotaxime (1.4%) were observed in humans. Moreover, the highest levels of 'microbiological' resistance to these critically important antimicrobials were detected in broiler meat (68% ciprofloxacin and 10.1% cefotaxime) and turkey meat (73.4% ciprofloxacin and 4.7% cefotaxime), suggesting an important contribution of the poultry production chain to the human burden [43]. In addition, it was reported that the highest levels of resistance to ciprofloxacin were more common in *S.* Enteritidis, *S.* Infantis and *S.* Kentuchy, three serotypes commonly associated with poultry meat [43]. Also, in the USA, in spite of a decreasing trend since 2009, cephalosporin's resistance levels are low in humans (2.5% ceftriaxone), but still high in turkey (9%) and retail chicken (20%), especially in *S.* Heidelberg a poultry-associated serotype [53].

In middle-income countries such as in the Asiatic continent, resistance to ciprofloxacin and extended-spectrum cephalosporins is currently a growing problem, with poultry products playing a potential role in its emergence [2,3]. High levels of *Salmonella* non-susceptible to ciprofloxacin (15%–48%) and cephalosporins (38% ceftriaxone) were observed in humans in Asian countries [54,55]. These data are in agreement with several studies that documented a high prevalence of resistance to fluoroquinolones (>22.5% ciprofloxacin) and cephalosporins (12.5% to >23.4% ceftriaxone and 26.6% ceftazidime) in poultry meat from South Korea and China [24,25]. In these countries the high rates of antimicrobial resistance detected reflect the levels of antimicrobial consumption in livestock, including for growth promotion, an area that remains largely unregulated [25,55,56]. Recent global estimates indicate that consumption of antibiotics in livestock (especially in poultry), which outranks the consumption by humans, will rise especially in middle-income countries. In these countries extensive farming systems will be replaced by large-scale intensive husbandry systems that routinely use antibiotics, to respond to the increasing demand for animal protein [56]. Therefore, international trade of contaminated breeding animals (poultry production depends on a pyramid-like breeding system), feed and poultry products with antibiotic-resistant *Salmonella* may contribute to the rapid worldwide spread of these bacteria, with impacts on human health.

A third indication of the significant impact on human health of antibiotic-resistant *Salmonella* contaminating these foodstuffs, is provided by diverse reports demonstrating their association with plasmid-mediated resistance determinants to cephalosporins and fluoroquinolones [49,51,57]. Moreover, several studies provide further evidence of transmission from poultry to humans of those clinically relevant MDR *Salmonella* clones [11,49,51,57]. A wide range of *Salmonella* serotypes (e.g. Enteritidis, Heidelberg, Infantis, Kentucky, Typhimurium, Virchow) frequently recovered from poultry (animal and food) have been associated with the worldwide dissemination of extended-spectrum β-lactamases, plasmid-encoded AmpC β-lactamases (AmpC), or plasmid-mediated quinolone resistance genes (Tables 2 and 3). By far the most common genes, found in poultry and/or poultry meat samples, associated with resistance to extended-spectrum cephalosporins, were those coding for extended-spectrum β-lactamases, CTX-M (e.g. CTX-M-1, -2, -9 and -15) and TEM-52 enzymes followed by AmpC-type CMY-2 (Table 2). For example, in several European countries, spread of *S.* Virchow producing CTX-M-2 or CTX-M-9 and *S.* Infantis producing TEM-52 has been found among poultry and humans [58–66]. Instead, *S.* Heidelberg, one of the

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TABLE 2. ***Salmonella*** **serotypes/clones carrying extended-spectrum and AmpC β-lactamases recovered from poultry and poultry products**

Salmonella serotype	ESBL or AmpC[a] enzymes (no. of isolates)	Source	Concomitant presence in humans[b]	Country(ies)/ year(s)	Non-β-lactam resistance phenotype[c]	Genetic element PL – Inc group	Reference(s)
Agona	CTX-M-1 (n=1)	Poultry (caecum)		The Netherlands/2006	SUL-TET	PL – I1	[65,66]
	CTX-M-2 (n=2)	Poultry		Brazil/2011	NR	PL – NR	[71]
		Turkey carcass		Brazil/2008	CIP-NAL-STR-SXT-TET	PL – P	[72]
	CTX-M-8 (n=3)	Poultry		Brazil/2008, 2010	NR	PL – NR	[71]
	TEM-52 (n=3)	Poultry		Belgium/2001, 2004	(–)	PL– I1	[62]
	CMY-2 (n=5)	Turkey		Germany/2005	(–) or NAL	PL – I1	[73]
Bareilly	ACC-1 (n=6)	Poultry	Yes	The Netherlands/ 2001–02	(–)	PL – NR	[74]
Blockley	TEM-52 (n=6)	Poultry, poultry meat	Yes	The Netherlands/ 2001–02	(–) or SPT-SUL-TMP	PL – NR	[74]
Brackenridge	CTX-M-14 (n=1)	Poultry		Brazil/2011	NR	PL – NR	[71]
Braenderup	ACC-1 (n=2)	Broilers		The Netherlands/2006	(–)	PL– NT	[65]
		Broiler		The Netherlands/2001	(–)	PL – NR	[74]
	SHV-12 (n=1)	Poultry		Italy/2006	(–)	PL – NR	[75]
Bredeney	CTX-M-1 (n=1)	Turkey		USA/2010	NR	PL – N	[76]
	CMY-2 (n=1)	Turkey		Canada/1999	SUL-TET	PL – A/C	[77]
Derby	TEM-52 (n=1)	Poultry		Belgium/2001	(–)	PL – I1	[62]
Enteritidis	CTX-M-2 (n=1)	Poultry		Brazil/2004	NR	NR	[71]
	CTX-M-9 (n=1)	Broiler		Spain/2000–04	STR-SUL-SXT-NAL-TET	NR	[60]
	CTX-M-14 (n=5)	Imported chicken meat		China/2004	NAL	PL – NR	[78]
		Poultry		China/2012–13	CHL-CIP-ENR-FFC-NAL-OLA- SXT-(AMK-GEN-LVX-TET)	PL – HI2, F, N or B/O	[70]
	CTX-M-15 (n=34)	Chicken meat, faeces or disease	Yes	Korea/2009	STR-SUL-TET-(GEN-NEO)	PL – FII	[79]
		Chicken		South Korea/2009–10	NR or GEN-STR-SUL-TET	PL – FII	[80]
	SHV-12 (n=2)	Broiler		Portugal/2012–13	CHL-CIP-SUL-NAL-TET	NR	[81]
		Poultry		Italy/2006	TET	NR	[75]
Emek	CMY-2 (n=1)	Poultry		Brazil/2011	NR	NR	[71]
Essen	CTX-M-15 (n=1)	Diseased chicken		Korea/2009	GEN-STR-SUL-TET	PL – FII	[79]
Gaminara	CTX-M-14 (n=1)	Poultry		Brazil/2009	NR	NR	[71]
Give	CTX-M-2 (n=1)	Poultry		Brazil/2009	NR	NR	[71]
Hadar	CTX-M-14 (n=1)	Poultry		Brazil/2005	NR	NR	[71]
Havana	CTX-M-1 (n=1)	Broiler		Portugal/2012–13	SUL-TET	NR	[81]
	CMY-2 (n=2)	Broiler		Portugal/2012–13	(–)	NR	[81]
Heidelberg	CTX-M-1 (n=1)	Broiler carcass		Portugal/2012–13	SUL-TMP	NR	[81]
	CTX-M-2 (n=14)	Poultry		Brazil/2009, 2011	NR	NR	[71]
		Chicken		Venezuela/2005–07, 2008	CIP-GEN	PL – NR	[82]
	CTX-M-14 (n=1)	Poultry		Brazil/2011	NR	NR	[71]
	SHV-2 (n=1)	Retail chicken meat		Canada/2007	GEN-STR-SUL	PL – I1	[83]
	CMY-2 (n=25)	Chicken or chicken retail	Yes	Canada/2001–04	CHL-GEN-KAN-STR-SUL-SXT-TET	PL – A/C, I1	[67]
		Chicken food		USA/2002, 2004, 2010	(CHL-GEN-KAN-STR-SUL-TET)	PL – A/C, I1	[31]
		Poultry		Brazil/2011	NR	NR	[71]
Indiana	CTX-M-14 (n=21)	Poultry		China/2012–13	ENR-NAL-SXT-(AMK-CHL-CIP-FFC-GEN-LVX-OLA-TET)	PL – HI2, A/C, F, N, P-1α, B/O	[70]
	CTX-M-24 (n=29)	Chicken		China/2008–09	AMK-CHL-FFC-GEN-NAL-OLA-RIF-STR-SXT-(TET)	PL – HI2	[84]
	CTX-M-65 (n=3)	Poultry		China/2012–13	CHL-NAL-OLA-SXT-(CIP-ENR-FFC-GEN-LVX-TET)	PL – HI2, F, N	[70]
	TEM-52 (n=1)	Broilers		The Netherlands/2006	(–)	PL – NT	[65]
	DHA-1 (n=3)	Chicken faeces		South Korea/2006–07	SUL-TMP-(APM-NAL-NEO-STR-TET)	PL – NR	[85]
Infantis	CTX-M-1 (n=2)	Poultry (caecum or unknown)		The Netherlands/2006	SUL-TMP	PL – I1	[65,66]
	CTX-M-2 (n=3)	Poultry		Brazil/2005	NR	NR	[71]
		Retail Chicken Products		Japan/2002-03	(–)	NR	[86]
	TEM-20 (n=2)	Retail Chicken Products		Japan/1997, 2003	(–)	NR	[86]
	TEM-52 (n=14)	Poultry	Yes	Belgium/2001–05	(–)	PL – I1	[62]
		Poultry (caecum or unknown)		The Netherlands/2006	(–)	PL – I1	[65,66]
		Broiler		Belgium/2004	(–)	PL – I1	[63]
		Chicken		Japan/2004–06	(–)	PL – NR	[87]
		Retail Chicken Products		Japan/2000, 2003	(–)	NR	[86]
	CMY-2 (n=48)	Retail Chicken Products		Japan/1997, 1999–2003	(–)	PL – NR	[86]
Kentucky	CTX-M-25/OXA-21 (n=1)	Turkey		Poland/2009	CIP-NAL	PL – A/C	[42]
	SHV-12 (n=3)	Whole chicken and chicken neck skin		Ireland/2008–09	CHL-SUL-TET	PL – NR	[88]
	CMY-2 (n=3)	Whole chicken		Ireland/2009	(–)	PL – NR	[88]
		Chicken meat		Germany/2005	STR	PL – I1	[73]
Kiambu	SHV-2 (n=1)	Abattoir chicken cecum		Canada/2006	STR-SUL-SXT	PL – I1	[83]
Livingstone	SHV-12 (n=9)	Poultry or turkey carcasse, poultry faeces, broiler faeces		Italy/2005-06	GEN-NAL-(STR-SUL)	NR	[75]
Llandoff	CTX-M-1 (n=1)	Poultry		France/2006	SUL-TET	PL – I1	[89]
Manhattan	CTX-M-2 (n=2)	Retail Chicken Products		Japan/2000, 2003	(–)	NR	[86]

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TABLE 2. Continued

Salmonella serotype	ESBL or AmpC[a] enzymes (no. of isolates)	Source	Concomitant presence in humans[b]	Country(ies)/ year(s)	Non-β-lactam resistance phenotype[c]	Genetic element PL – Inc group	Reference(s)
	CTX-M-15 (n=4)	Retail Chicken Products		Japan/2002, 2003	(–)	NR	[86]
	TEM-52 (n=4)	Retail Chicken Products		Japan/2000, 2002–03	(–)	NR	[86]
	SHV-12 (n=2)	Retail Chicken Products		Japan/2002, 2003	(–)	NR	[86]
	CMY-2 (n=2)	Retail Chicken Products		Japan/2002	(–)	PL – NR	[86]
Minnesota	CTX-M-2 (n=1)	Poultry		Brazil/2009	NR	NR	[71]
	CTX-M-8 (n=1)	Poultry		Brazil/2008	NR	NR	[71]
	CTX-M-14 (n=1)	Poultry		Brazil/2010	NR	NR	[71]
	CMY-2 (n=2)	Poultry		Brazil/2011	NR	NR	[71]
Newport	CTX-M-2 (n=1)	Poultry		Brazil/2008	NR	NR	[71]
Ouakam	CTX-M-1 (n=6)	Turkey		USA/2011	NR	PL – N	[76]
Panama	CTX-M-1 (n=1)	Poultry		Brazil/2009	NR	NR	[71]
Poona	CTX-M-2 (n=1)	Poultry		Brazil/2009	NR	NR	[71]
	CTX-M-8+CTX-M-14 (n=1)	Poultry		Brazil/2005	NR	NR	[71]
Rissen	CMY-2 (n=1)	Poultry		Brazil/2005	NR	NR	[71]
Saintpaul	CMY-2 (n=1)	Poultry		Brazil/2011	NR	NR	[71]
Schwarzengrund	CTX-M-2 (n=10)	Turkey, chicken, chicken carcass		Brazil/2008	CIP-NAL-STR-TET	PL – P	[72]
Senftenberg	CTX-M-15 (n=1)	Chicken		South Korea/2009	NR	PL – FII	[80]
Typhimurium	CTX-M-1 (n=1)	Poultry meat		Germany/2006	KAN-NEO-STR-SUL-SXT-TET-TMP	PL – I1	[73]
	CTX-M-2 (n=10)	Poultry		Brazil/2004–05, 2007	NR	NR	[71]
		Poultry	Yes	Brazil/2003–04	SUL-SXT-TET	PL – NR	[90]
	CTX-M-8 (n=1)	Poultry		Brazil/2011	NR	NR	[71]
	CTX-M-14 (n=1)	Poultry		Brazil/2005	NR	NR	[71]
	CTX-M-15 (n=1)	Broiler		Belgium/2007	(–)	PL – I1	[63]
	TEM-52 (n=4)	Poultry	Yes	Belgium/2002, France/2004	(–)	PL – I1	[62]
		Poultry		The Netherlands/2001–02	SPT-SUL-TMP-(NEO-TET)	NR	[74]
	TEM-52+SHV-12 (n=1)	Poultry meat		The Netherlands/2002	(–)	NR	[74]
Virchow	CTX-M-2 (n=12)	Poultry or poultry product		Belgium/2000–01, 2003	NAL-SUL-TET-TMP-(STR)	PL – HI2	[61]
		Poultry		Ireland/UN	SUL-TET-TMP	PL – P	[77]
		Broiler		Belgium/2001, 2004	STR-SUL-TET-TMP	PL – HI2	[63]
		Poultry	Yes	Belgium-France/2000–03	NAL-SUL-TMP-(STR-TET)	PL – NR	[59]
	CTX-M-2+SHV-2+TEM-1 (n=1)	Broiler		The Netherlands/2002	NAL-SPT-SUL-TET- TMP	NR	[74]
	CTX-M-9 (n=8)	Chicken faeces		France/2002–03	KAN-NAL-SPT-STR-SUL-TET-TMP	PL – NR	[58]
		Chicken faeces and retail chicken meat	Yes	France/2002–03, Spain/2000	NAL-SPT-STR-SUL-TET-TMP-(KAN)	PL – HI2	[61]
		Broiler	Yes	Spain/2000–04	STR-SUL-SXT-NAL-(TET)	NR	[60,64]
	CTX-M-32 (n=2)	Poultry		Greece/2001	CHL-KAN-STR-SUL-TET-TMP	PL – NR	[91]
	TEM-52 (n=1)	Poultry		The Netherlands/2002	SPT-STR-SUL-TMP	NR	[74]
Weslaco	CTX-M-8 (n=1)	Poultry		Brazil/2009	NR	NR	[71]
35:c:1,2	SHV-12 (n=5)	Poultry	Yes	Senegal/2000	CHL-GEN-TET-TOB	NR	[92]

Antimicrobial abbreviations: AMK, amikacin; APM, apramycin; CHL, chloramphenicol; CIP, ciprofloxacin; ENR, enrofloxacin; FFC, florfenicol; GEN, gentamicin; KAN, kanamycin; LVX, levofloxacin; NAL, nalidixic acid; NEO, neomycin; OLA, olaquindox; RIF, rifampicin; SPT, spectinomycin; STR, streptomycin; SUL, sulphonamides compound; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; TMP, trimethoprim; TOB, tobramycin; NR, not reported; NT, plasmids that were not typable with the scheme used; PL, plasmid.
[a]Only references with full-characterized extended-spectrum β-lactamases /AmpC genes were considered.
[b]'Yes', clones or serotypes simultaneoulsy detected in poultry and humans in the same study.
[c](–), Non-β-lactam resistance phenotype was not detected using the tested antibiotics and according to the susceptibility criteria adopted. Variable phenotypes were present between curved brackets.

top MDR serotypes in both poultry and humans (frequently associated with invasive infections) in North America, presented increasing resistance to cephalosporins related with CMY-2 [31,67]. In fact, transmission of these most reported genes in both human and poultry has been associated with diverse plasmid families, such as IncI1 (e.g. $bla_{CTX-M-1}$, bla_{TEM-52} and bla_{CMY-2}), IncA/C (e.g. bla_{CMY-2}) or IncHI2 (e.g. $bla_{CTX-M-2}$ and $bla_{CTX-M-9}$) (Table 2). Recently, plasmid-mediated carbapenem-resistant bacteria from food-animals have been reported, alerting us to a new public health problem with indefinable risks [51]. One of the first reports was on a poultry farm in Germany where a bla_{VIM-1} gene was detected on *S.* Infantis [68]. Besides the worldwide resistance to clinically important β-lactams, plasmid-mediated quinolone resistance mechanisms, which typically confer decreased susceptibility to ciprofloxacin, have been widely reported in *Salmonella* [57]. Among them, Qnr proteins (e.g. QnrB2, QnrB19, QnrS1) have been commonly described in different serotypes and geographic locations, with the aminoglycoside acetyltransferase AAC(6′)-Ib-cr and the efflux pump OqxAB associated with *Salmonella* serotypes recovered from Asian poultry and poultry meat (Table 3). In fact, poultry seems to be an important vehicle of non-typhoidal *Salmonella* carrying plasmid-mediated quinolone resistance genes, highlighting the role of food-producing

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TABLE 3. ***Salmonella*** **serotypes/clones carrying plasmid-mediated quinolone resistance genes recovered from poultry and poultry products**

Salmonella serotype	PMRQ[a] mechanism (no. of Isolates)	Source	Country(ies)/year(s)	Antibiotic resistance phenotype[b]	Genetic element PL – Inc group	Reference(s)
Agona	QnrB2 (n=1)	Turkeys	Germany/NR	NR	NR	[69]
Braenderup	QnrD (n=2)	Fowls	Spain/NR	NR	NR	[69]
Dabou	QnrD (n=1)	Fowls	Spain/NR	NR	NR	[69]
Derby	QnrB2 (n=56)	Fowls or turkeys	Spain/NR	NR	NR	[69]
Enteritidis	QnrB2 (n=1)	Fowls or turkeys	Spain/NR	NR	NR	[69]
	QnrB10/B19 (n=1)	Laying hen flock	Poland/2009	CIP-NAL	NR	[93]
	QnrD (n=3)	Fowls	Spain/NR	NR	NR	[69]
	QnrS1/S3 (n=2)	Broiler meat, broiler flock – faeces	Poland/2008–09	AMP-CIP-(STR-TET)	NR	[93]
	OqxAB (n=3)	Poultry	China/2012–13	AMP-CHL-CIF-CIP-ENR-FFC-NAL-OLA-SXT-(GEN-LVX-TET)	PL – HI2, F, N, B/O	[70]
	OqxAB + AAC(6′)-Ib-cr (n=1)	Poultry	China/2012–13	AMK-AMP-CHL-CIF-CIP-CTX-ENR-FFC-GEN-LVX-NAL-OLA-SXT-TET	PL – HI2	[70]
Give	QnrB19 (n=1)	Imported turkey meat from Brazil	Finland/NR	NR	NR	[69]
Hadar	QnrB2 (n=2)	Fowls or turkeys	Spain/NR	NR	NR	[69]
	QnrB5 (n=4)	Imported turkey meat	Germany/2007	STR-TET	NR	[94]
	QnrB19 (n=15)	Fowls, turkeys	Germany, Denmark/NR	NR	NR	[69]
Havana	QnrB2 (n=3)	Poultry	Portugal/2009–10	(–) or NAL	PL – L/M	[95]
	QnrB19 (n=1)	Poultry	Portugal/2009–10	(–)	PL – HI2	[95]
Heidelberg	QnrB19 (n=2)	Chicken	Venezuela/2005–07, 2008	CIP-GEN	PL – NR	[82]
Indiana	OqxAB (n=50)	Poultry	China/2012–13	AMP-CIF-NAL-SXT-(AMK-CAZ-CHL-CIP-CTX-ENR-FFC-GEN-LVX-OLA-TET)	PL – HI2, F, N, P-1α, B/O	[70]
		Chicken	China/2008–09	AMK-CHL-FFC-GEN-NAL-OLA-RIF-STR-SXT-(TET)	PL – HI2	[84]
	OqxAB + AAC(6′)-Ib-cr (n=3)	Poultry	China/2012–13	AMP-CHL-CIF-NAL-SXT-(CIP-CTX-ENR-FFC-GEN-LVX-OLA-TET)	PL – HI2, A/C, N	[70]
Infantis	QnrS1 (n=1)	Chicken	Germany/2004	AMP	NR	[96]
	QnrB19 (n=1)	Retail chicken	Colombia/2004	KAN-NAL-NEO-STR-TET	PL – ColE like	[97]
Kentucky	QnrS1 (n=1)	Chicken	The Netherlands/NR	NR	PL – N	[98]
London	QnrB2 (n=22)	Fowls or turkeys	Spain/NR	NR	NR	[69]
Mbandaka	QnrB19 (n=1)	Poultry	Portugal/2009–10	NAL	PL – HI2	[95]
Montevideo	QnrB2 (n=3)	Fowls or turkeys	Spain/NR	NR	NR	[69]
	QnrD (n=6)	Fowls, turkeys	Italy/NR	NR	NR	[69]
Newport	QnrB5 (n=3)	Imported turkey meat	Poland/2007	(–)	NR	[94]
	QnrS1/S3 (n=12)	Broiler (meat, flock), turkey meat, goose flock – faeces, duck flock – faeces	Poland/2008–11	CIP-(AMP-CHL-FFC-KAN-STR-SUL-TET-NAL)	NR	[93]
	QnrB19 (n=3)	Turkeys	Denmark/NR	NR	NR	[69]
Ohio	QnrD (n=5)	Fowls	Spain/NR	NR	NR	[69]
Saintpaul	QnrS1 (n=15)	Imported turkey meat	Germany/2007–08 Poland/2008	AMC-AMP-STR-TET-(CPD-CHL)	NR	[94]
		Turkeys	Germany, Denmark/NR	NR	NR	[69]
Typhimurium	QnrA1 (n=1)	Turkeys	Germany/NR	NR	NR	[69]
	QnrD (n=2)	Fowls	Spain/NR	NR	NR	[69]
	OqxAB (n=2)	Chicken	China/2007–09	OLA-FFC-SMX-(AMP-CHL-CIF-GEN-NAL-TET)	PL – HI2, F	[99]
	OqxAB + AAC(6′)-Ib-cr (n=8)	Chicken, duck	China/2009–10	NAL-OLA-SMX-(AMP-CHL-CIF-ENR-FFC-GEN-TET)	PL – HI2	[99]
	OqxAB + AAC(6′)-Ib-cr + QnrS1 (n=1)	Duck	China/2010	AMP-CHL-CIF-FFC-GEN-NAL-OLA-SMX-TET	PL – HI2	[99]
Virchow	QnrS1	Chicken carcass	Korea/2002	AMP-CEF-STR-SUL- TET-TMP	N	[100]
		Chicken	UK/2004-05	AMP-CIP	N	[101]
		Chicken	Turkey/2005	AMP-NAL	PL – NT	[102]

Antimicrobial abbreviations: AMC, amoxicillin/clavulanate; AMK, amikacin; AMP, ampicillin; CAZ, ceftazidime; CEF, cefalotin; CHL, chloramphenicol; CIF, ceftiofur; CIP, ciprofloxacin; CPD, cefpodoxime; CTX, cefotaxime; ENR, enrofloxacin; FFC, florfenicol; GEN, gentamicin; KAN, kanamycin; LVX, levofloxacin; NAL, nalidixic acid; NEO, neomycin; OLA, olaquindox; RIF, rifampicin; STR, streptomycin; SUL, sulphonamides compound; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; TMP, trimethoprim; NR, not reported; NT, plasmids that were not typeable with the scheme used; PL, plasmid.
[a]Only references with full-characterized plasmid-mediated quinolone resistance genes were considered.
[b](–), Antibiotic resistance phenotype was not detected using the tested antibiotics and according to the susceptibility criteria adopted. Variable phenotypes were present between curved brackets.

animals, including the animal/food trade, in its dissemination [69,70].

In addition to the therapeutic consequences, antimicrobial resistance acquisition to agents commonly used in livestock production has been associated with the spread of particular *Salmonella* clones in the poultry production environment, which might contribute to their association with human diseases. Interestingly, the high-level resistance to ciprofloxacin (due to

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gyrA/*par*C mutations) and other antibiotics (amoxicillin, streptomycin, spectinomycin, gentamicin, sulfamethoxazole and tetracycline) [39–43] of the previously mentioned *S.* Kentucky ST198-X1-SGI1 strain might also have contributed to its clonal expansion. In the EU, several *S.* Stanley outbreaks linked with the consumption of turkey meat products have been reported, with the clone presenting resistance to nalidixic acid and decreased susceptibility to ciprofloxacin. Additionally, in the recent outbreak in Austria three strains also presented resistance to gentamicin and cephalosporins (CTX-M-15) [36,38]. Several successful *S.* Infantis clones of broiler origin are also characterized by multidrug resistance profiles, including nalidixic acid, tetracyclines, sulphonamides or nitrofurans [26,35,46], antibiotics frequently used in livestock production, which could contribute to their co-selection in the poultry industry.

Data compiled here, highlight the role of the poultry production chain (including poultry meat) as a reservoir of epidemic MDR clones or genes conferring resistance to critical antibiotics, which might spread to humans through the food chain. Globalization of food-animal production is currently posing a major challenge in antimicrobial resistance of zoonotic bacteria like non-typhoidal *Salmonella* with a consequent involvement of human health. In particular in the poultry production pyramid it is crucial to restrict the global use of antimicrobials to minimize the selection of resistant *Salmonella* (from the top of the poultry production pyramid and within flocks), and also to control the spread of MDR epidemic clones by improving biosecurity measures from the farm (e.g. hygiene high standards, vaccination) throughout the food chain (e.g. slaughtering and processing of poultry meat) and control of animal/food trade.

Conclusions

In the last decade, *Salmonella* control programmes, mainly targeting poultry production, have led to a significant reduction in salmonellosis in different countries, including in the EU. This positive effect has been associated with a shift in *Salmonella* serotypes of poultry and human disease, associated with a marked reduction on *S.* Enteritidis and the increase of less common serotypes, driven by the dissemination of particular clones carrying features favouring host (poultry/human) adaptability and frequently MDR.

Poultry has been reported as a source of non-typhoidal *Salmonella* resistant to clinically relevant antibiotics with a remarkable higher incidence reported for middle-income countries, which due to food trade globalization can dramatically challenge worldwide the treatment of severe salmonellosis cases.

Integrated surveillance (collaboration between human health, food safety and animal health—the 'One Health' approach) and containment strategies (including farms, retail, catering and consumers) to minimize contamination and reduce the transmission of *Salmonella* (including epidemic clones) along the food chain (from primary production to consumption) are mandatory on a global scale, in particular in the poultry production chain. In addition, continuous surveillance of *Salmonella* resistance levels globally is critical for clinicians to support the best salmonellosis treatment choices, avoid treatment failures, particularly for patients that would benefit from empirical antibiotic treatment.

Continuous monitoring to detect the emergence of any serotype or new clone along the food chain is of critical importance for public health, warning of the emergence of new *Salmonella* food safety risks, involving foodstuffs like poultry meat, which is one of the most consumed and increasing globally traded meat products.

Transparency Declaration

The authors declare no conflict of interest.

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II. Protocols optimized for assessment of metal and biocide susceptibility by genotypic and/or phenotypic methods

Polymerase chain reaction (PCR) assays for detecting metal tolerance genes

TABLE 1 - Primers used in PCR assays to search acquired metal tolerance genes

Operon or cluster	Target Gene	Gene coding for	Primer	Primer sequence (5'-3')[a]	Nucleotide position (GenBank accession no.)	Reference
sil	*silA*	Silver inner-membrane proton/cation antiporter	silA_Fw silA_Rv	GCAAGACCGGTAAAGCAGAG CCTGCCAGTACAGGAACCAT	8936-8955 9871-9852 **(AF067954.1)**	Mourão *et al.*, 2015
	silC	Outer membrane protein	silC_4090_F silC_4470_R	CGGGCTGGCGHAMCTTTTTTG CCAGTTGCTGRYTGAAATARC	4090-4110 4470-4450 (**BX664015.1**)	This study
	silE	Silver/copper periplasmic binding protein	silE_1105_F silE_1368_R	GTTCGTCATGGTYTCATGAGC GTACTYCCCCGGACATCACTAATT	1105-1125 1368-1345 (**AF067954.1**)	Mourão *et al.*, 2015
	silP	P-type cation ATPase	silP_11882_F silP_12405_R	GGCGATAAGCTCCGCATCAGA TCCACTTTTTCAAGACGCTCA	11882-11902 12405-12385 (**BX664015.1**)	Kremer *et al.*, 2014
	silR	Transcriptional regulator responder	silR_3244_F silR_3572_R	CCCTGATGGCGAAKGAAGAA AACGGCTGGGATATCRTSCG	3244-3263 3572-3553 (**BX664015.1**)	Kremer *et al.*, 2014
	silS	Membrane kinase sensor	silS_2279_F silS_3019_R	GCGGGTAAARACATCCTCAAT CTGACCTTTTTTATCAGCCTG	2279-2299 3019-2999 (**BX664015.1**)	Kremer *et al.*, 2014
pco	*pcoA*	Multicopper oxidase	pcoA_978_F pcoA_1481_R	CTCGCGGATGTCAGTGGCTACACCT ATCCGGAAGGTCAGCACCGTCCATAGAC	978-1002 1481-1454 (**X83541.1**)	Mourão *et al.*, 2015
	pcoD	Copper inner membrane pump	pcoD_F pcoD_R	CTGGCCACACTTGCCTGGGG CACGCTACGGCGCCCAGAAT	3801-3820 4300-4281 (**X83541.1**)	Mourão *et al.*, 2015
	pcoE	Periplasmic chaperone	pcoE_6585_F pcoE_7057_R	CCTGGTTCCTGGAGTGATGA TGACCATTATTCGCCTTCT	6585-6604 7057-7039 (**X83541.1**)	This study
	pcoR	Regulator of *pcoABCD* cluster	pcoR_4460_F pcoR_4928_R	AGGCTATCAGGCCGATCTCT CCAGACCAGGGACGAGATAA	4460-4478 4928-4909 (**X83541.1**)	This study
	pcoS	Sensor of *pcoABCD* cluster	pcoS_5182_F pcoS_6236_R	TAATCAGGACCGCGATTTTC CACTGTCACGCTCAAGGTGT	5182-5201 6236-6217	This study

Operon or cluster	Target Gene	Gene coding for	Primer	Primer sequence (5'-3')[a]	Nucleotide position (GenBank accession no.)	Reference
					(**X83541.1**)	
tcr	*tcrB*	Copper export ATPase	tcrB_F tcrB_R	CATCACGGTAGCTTTAAGGAGATTTTC ATAGAGGACTCCGCCACCATTG	4954–4980 5616–5595 **(AY048044.2)**	Hasman *et al.*, 2006
mer	*merA*	Mercuric reductase	merA_1F merA_5R	ACCATCGGCGGCACCTGCGT ACCATCGTCAGGTAGGGGAAC	2140-2159 3377-3357 (**K03089.1**)	Liebert *et al.*, 1997
ars	*arsB*	Arsenite transmembrane pump	arsB_Fw arsB_Rv	AGTGAAAGACAGACGAAGCG GGCAGATAGTGTGGAATGCG	159735-159754 160870-160851 (**BX664015.1**)	García-Fernández *et al.*, 2012
ter	*terF*	Tellurite resistance protein	terF_Fw1 terF_Rv	ATAGCACTGGATCGTGTTCC TTCATCGATCCACGGTCTG	80174-80193 81163-81145 (**BX664015.1**)	This study

[a]H = A, C or T; K = G or T; M = A or C; R = A or G; S = G or C; Y = C or T.

TABLE 2 - Conditions used for the PCR assays to search acquired metal tolerance genes

Operon or cluster	Target Gene	Primer	Taq Polymerase kit used (Vfinal = 25 µl)	Amplification conditions	Annealing temperature (ºC)	Amplicon size (bp)
sil	*silA*	silA_Fw silA_Rv	Buffer 0,5x 2mM of MgCl2 200 µM of each dNTP 0,2 µM of each Primer KAPA Taq DNA Polymerase 1U	94ºC - 10 minutes (1 cycle) 94º - 1 minute **T_Aº** - 1 minute 72º - 2 minutes (30 cycles) 72º - 10 minutes (1 cycle)	59	936
	silC	silC_4090_F silC_4470_R			60	381
	silE	silE_1105_F silE_1368_R			62	264
	silP	silP_11882_F silP_12405_R			60	524
	silR	silR_3244_F silR_3572_R			62	329
	silS	silS_2279_F silS_3019_R			60	741
pco	*pcoA*	pcoA_978_F pcoA_1481_R	Buffer 1x 1,5mM of MgCl2	95ºC – 10 minutes (1 cycle) 94º - 30 seconds	60	504

Operon or cluster	Target Gene	Primer	Taq Polymerase kit used (Vfinal = 25 µl)	Amplification conditions	Annealing temperature (ºC)	Amplicon size (bp)
	pcoD	pcoD_F pcoD_R	200 µM of each dNTP 0,2 µM of each Primer	**T_Aº** - 30 seconds 72º - 30 seconds (30 cycles)	55	500
	pcoE	pcoE_6585_F pcoE_7057_R	PROMEGA Go Taq® DNA Polymerase 0,5U	72º - 10 minutes (1 cycle)	58	473
	pcoR	pcoR_4460_F pcoR_4928_R			58	469
	pcoS	pcoS_5182_F pcoS_6236_R			63	1055
tcr	*tcrB*	tcrB_F tcrB_R	Buffer 1x 1,5 mM of $MgCl_2$ 200 µM of each dNTP 0,125 µM of each Primer PROMEGA Go Taq® DNA Polymerase 1,25U	95ºC- 10 min (1 cycle) 94ºC-30 seconds **T_A**ºC-45 seconds 72ºC-45 seconds (25 cycles) 72ºC-10 minutes (1 cycle)	55	990
mer	*merA*	merA_1F merA_5R	Buffer 1x 1,5mM of $MgCl_2$ 200 µM of each dNTP 0,2 µM of each Primer KAPA Taq DNA Polymerase 0,5U	95ºC – 10 minutes (1 cycle) 94º - 30 seconds **T_Aº** - 1 minute 72º - 2 minutes (30 cycles) 72º - 10 minutes (1 cycle)	65	1238
ars	*arsB*	arsB_Fw arsB_Rv	Buffer 1x 1,5mM of $MgCl_2$ 200 µM of each dNTP 0,2 µM of each Primer PROMEGA Go Taq® DNA Polymerase 1U	95ºC – 10 minutes (1 cycle) 94º - 30 seconds **T_Aº** - 30 seconds 72º - 30 seconds (25 cycles) 72º - 10 minutes (1 cycle)	60	1136
ter	*terF*	terF_Fw1 terF_Rv	Buffer 1x 1,5mM of $MgCl_2$ 200 µM of each dNTP 0,2 µM of each Primer PROMEGA Go Taq® DNA Polymerase 0,5U	95ºC – 10 minutes (1 cycle) 94º - 1 minute **T_Aº** - 1 minute 72º - 2 minutes (30 cycles) 72º - 10 minutes (1 cycle)	60	990

Appendix references:

Garcia Fernandez A, Cloeckaert A, Bertini A, Praud K, Doublet B, Weill FX*, et al.* Comparative analysis of IncHI2 plasmids carrying $bla_{CTX\text{-}M\text{-}2}$ or $bla_{CTX\text{-}M\text{-}9}$ from *Escherichia coli* and *Salmonella enterica* strains isolated from poultry and humans. Antimicrob Agents Chemother. 2007;51(11):4177-80.

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Determination of copper sulphate and silver nitrate minimum inhibitory concentrations (MICs) by the agar dilution method in aerobic and anaerobic atmospheres

Strains:

Day 1

a) Prepare and isolate test cultures and quality control strain cultures on CLED (MIC_{CuSO4}) or Mueller-Hinton Agar/MHA (MIC_{AgNO3}) and check purity before.

Control strains:

For CuSO4 susceptibility assays

- *Enterococcus faecium* BM4105RF*
- *Escherichia coli* ED8739 (plasmid pRJ1004 with *pco* cluster) – offered by Jill Williams

For AgNO3 susceptibility assays

- *S. aureus* ATCC 29213*
- *Escherichia coli* J53 (plasmid pMG101 with *sil* cluster) -offered by Christopher Randall

* Both negative for all genes tested (*pcoD*$^-$, *silA*$^-$, *tcrB*$^-$, *cueO*$^-$)

TABLE 3 – MICs values of quality control strains to CuSO4 and AgNO4 in aerobic (with/without induction) and anaerobic atmospheres

Control strains	O_2 environment	*sil* genes	*pco* genes	MIC_{CuSO4} (mM)	MIC_{AgNO3} (mM)
Enterococcus faecium BM4105RF	Aerobic Anaerobic	(-)	(-)	8–12 2–4	
Escherichia coli ED8739 (pRJ1004)	Aerobic Anaerobic	(+)	(+)	32–36 16–20	
S. aureus ATCC 29213	Aerobic Induction *sil* Anaerobic	(-)	(-)		0.16–0.25 0.16–0.25 0.32–0.5
Escherichia coli J53 (pMG101)	Aerobic Induction *sil* Anaerobic	(+)	(+)		> 3 >3 > 3

Material:

Day 2

- Prepare first Mueller-Hinton Agar 2, cation adjusted (Biomérieux – Ref. 51075) following manufacturer's instructions. We will need 25 mL of MHA per plate to prepare all the concentrations in the range to test and to prepare control plates for growth controls ($MHA_{initial}$ and MHA_{final}).
- Autoclave the MHA at 121º C for 15 minutes.
- Cool to 60º C in water bath before aseptically add metal solutions.
- Pre-warm 50, 100 mL or 250 mL of measurement glasses (for mixing and pouring) in 60º C water bath until needed. We will need one Erlenmeyer flask for each concentration and graduated cylinders.

- After cooling down the media check pH using a pH meter, accordingly to the metal (7.2 – copper sulphate; 7.4 – silver nitrate) at room temperature before adding the metal solutions.

  NOTE: We have **three systems** for MIC's determination: 2 systems of 21 wells and 1 system of 17 wells.

  We will need the following concentrations for each metal:

1. **Cu^{+}/Cu^{2+} (aerobic and anaerobic conditions)**

0.25 mM – 0.5 mM – 1 mM - 2 mM – 4 mM – 8 mM – 12 mM – 16 mM – 20 mM – 24 mM – 28 mM – 32 mM – 36 mM (**aerobic**)
0.25 mM – 0.5 mM – 1 mM - 2 mM – 4 mM – 8 mM – 12 mM – 16 mM – 20 mM – 24 mM – 28 mM – 32 mM – 36 mM (**anaerobic**)
Mueller-Hinton Agar for one system = (13 concentrations aerobic + 13 concentrations anaerobic + 4 plates for $MH_{initial}$ and MH_{final}) x 25 mL agar = 750 mL MHA. Prepare MHA in excess (e.g. 900 mL).

2. **Ag^{2+} (aerobic with/without induction and anaerobic conditions)**

0.025 mM – 0.06 mM – 0.08 mM – 0.125 mM – 0.16 mM – 0.25 mM – 0.32 mM – 0.5 mM – 0.75 mM – 1 mM – 1.5 mM – 3 mM (**aerobic with/without induction**)

0.025 mM – 0.06 mM – 0.08 mM – 0.125 mM – 0.16 mM – 0.25 mM – 0.32 mM – 0.5 mM – 0.75 mM – 1 mM – 1.5 mM – 3 mM (**anaerobic**)

Mueller-Hinton Agar for one system = (12 concentrations aerobic + 12 concentrations anaerobic + 4 plates for $MH_{initial}$ and MH_{final}) x 25 mL agar = 700 mL MHA. Prepare MHA in excess (e.g. 900 mL).

Metal solutions:

…Day 2

The recommendation is preparing stock solutions from the compounds at least 10X the highest concentration of the range. Copper sulphate is prepared at 1M stock solution and silver nitrate at 10 mM stock solution in ultra-pure distilled water (Ex: if the highest concentration to test is 1mM the stock solution should be prepared at least at 10mM). See section above.

MW ($CuSO_4$) = 159.61 g/mol
MW ($AgNo_3$) = 169.87 g/mol

SAFETY- WEIGHING OF THE POWDER NEEDS TO BE DONE IN A HIGH SAFETY FUME HOOD WITH DOUBLE GLOVES) AND VERY CAREFULLY DUE TO THE POWDER TOXICITY! ADD WATER AS SOON AS POSSIBLE TO REDUCE CHANCES OF POWDER INHALATION!

Preparation of plates with copper sulphate, pH = 7.2

4 systems

PROCEDURE

A. Prepare 25 ml of the stock solution of $CuSO_4$ at 1M

$$c = \frac{n}{v} \leftrightarrow 1M = \frac{n}{0.025\ L} \leftrightarrow n = 0.025\ mol$$

$$n = \frac{m}{Mw} \leftrightarrow 0.025\ mol = \frac{m}{159.61} \leftrightarrow m = 3.99025\ g + 25\ ml\ of\ destilled\ water$$

B. <u>Make 2-fold dilutions – in 2 orders</u>

1.

24 mM – From 200 mL MH Agar, discard 4.8 mL of agar and add 4.8 mL of 1M Copper Sulphate stock solution mix and adjust pH to 7.2.

12 mM – Mix 50 ml 24 mM with 50 ml of MHA, mix and adjust pH to 7.2.

<u>See the first example to apply to the rest of the concentrations</u>:

$Cc\ x\ Vc = Cd\ x\ Vd \ \leftrightarrow 1M\ x\ Vc = 0.024\ M\ x\ 200\ mL \ \leftrightarrow Vc = \mathbf{4.8\ mL}$

2.

16 mM – From 200 mL MH Agar, discard 3.2 mL of agar and add 3.2 mL of 1M Copper Sulphate stock solution mix and adjust pH to 7.2.

8 mM – Mix 100 ml 16 mM with 100 ml of MHA, mix and adjust pH to 7.2.

4 mM – Mix 100 ml 8 mM with 100 ml of MHA, mix and adjust pH to 7.2.

2 mM – Mix 100 ml 4 mM with 100 ml of MHA, mix and adjust pH to 7.2.

1 mM – Mix 100 ml 2 mM with 100 ml of MHA, mix and adjust pH to 7.2.

0.5 mM – Mix 100 ml 1 mM with 100 ml of MHA, mix and adjust pH to 7.2.

0.25 mM – Mix 50 ml 0.5 mM with 50 ml of MHA, mix and adjust pH to 7.2.

Separately prepare:

20 mM – From 100 mL MH Agar, discard 2.0 mL of agar and add 2.0 mL of 1M Copper Sulphate stock solution mix and adjust pH to 7.2.

28 mM – From 100 mL MH Agar, discard 2.8 mL of agar and add 2.8 mL of 1M Copper Sulphate stock solution mix and adjust pH to 7.2.

32 mM – From 100 mL MH Agar, discard 3.2 mL of agar and add 3.2 mL of 1M Copper Sulphate stock solution mix and adjust pH to 7.2.

36 mM – From 100 mL MH Agar, discard 3.6 mL of agar and add 3.6 mL of 1M Copper Sulphate stock solution mix and adjust pH to 7.2.

C. Prepare all dilutions using $CuSO_4$ at recommended concentrations as follows:

Concentration (mM)	Order 1	Order 2	Volume of copper sulphate (ml)	Volume of MHA (ml)
$MH_{initial}$ + MH_{final}		Separately	0 mL	2 x 100 mL
2 x 0 mM				
0.25 mM		9	50 ml 0.5 mM agar	50 ml
0,5 mM		8	100 ml 1 mM agar	100 ml
1 mM		7	100 ml 2 mM agar	100 ml
2 mM		6	100 ml 4 mM agar	100 ml
4 mM		5	100 ml 8 mM agar	100 ml
8 mM		4	100 ml 16 mM agar	100 ml
12 mM	2		50 ml 24 mM agar	50 ml
16 mM		3	3.2 ml stock solution at 1M	200 ml
20 mM		Separately	2.0 ml stock solution at 1M	100 ml
24 mM	1		4.8 ml stock solution at 1M	200 ml
28 mM		Separately	2.8 ml stock solution at 1M	100 ml
32 mM		Separately	3.2 ml stock solution at 1M	100 ml
36 mM		Separately	3.6 ml stock solution at 1M	100 ml

Preparation of plates with silver nitrate, pH = 7.4

4 systems

PROCEDURE

D. Prepare 90 ml of the stock solution of $AgNO_3$ at 10 mM

$$c = \frac{n}{v} \leftrightarrow 0.010M = \frac{n}{0.090\ L} \leftrightarrow n = 0.0009\ mol$$

$$n = \frac{m}{Mw} \leftrightarrow 0.0009\ mol = \frac{m}{169.87} \leftrightarrow m = 0.152883\ g + 90\ ml\ of\ destilled\ water$$

E. Prepare 200 ml of a solution $AgNO_3$ at 1 mM in Mueller-Hinton Agar

$$Cc\ x\ Vc = Cd\ x\ Cv \leftrightarrow 10\ mM\ x\ Vc = 1\ mM\ x\ 200\ ml$$

$$Vc = 20\ ml\ of\ the\ 10\ mM\ silver\ solution + 180\ ml\ of\ MHA, adjust\ pH\ to\ 7.4$$

F. Prepare all dilutions using mix of agar at 1 mM and 10 mM as follows:

Concentration (mM) of stock solution	Volume of the $AgNO_3$ stock solution (ml)	Volume of Mueller-Hinton Agar (ml)	Final concentration (mM)
			$MH_{initial}$ + MH_{final}
0	0	2 x 100	**2 x 0 mM**
1	2.5	97.5	**0.025 mM**
1	6	94	**0.06 mM**
1	8	92	**0.08 mM**
1	12.5	87.5	**0.125 mM**
1	16	84	**0.16 mM**
1	25	75	**0.25 mM**
1	32	68	**0.32 mM**
1	50	50	**0.5 mM**
10	7.5	92.5	**0.75 mM**
10	10	90	**1 mM**
10	15	85	**1.5 mM**
10	30	70	**3 mM**

Preparation of plates:

…Day 2

- Distribute Mueller-Hinton Agar with metals (Cu or Ag) into Petri dishes at depth of about 3 to 4 mm (±25ml each), mark metal concentration on plates inside LAF bench.
- Plates must solidify at room temperature, and must be used in the same day.
- Evaluate potency and stability with control strains in all rounds of MIC testing – all the control strains are in the first page.
- Prepare 2 ($MH_{initial}$ and MH_{final}) control plates without metal solution as growth controls for each round.

Inoculum preparation:

…Day 2

- Prepare a suspension at 0.5 McFarland (1-2 x 10^8 CFU/ml) in 2 mL of sterile saline (NaCl) using a nephelometer.
- Dilute 1:10 in sterile saline to approximated 10^7 CFU/ml (100 µl of the former suspension at 0.5 McF + 900 µl saline) in 1.5 mL Eppendorf tubes before adding the suspension to the inoculum replicator block.

Inoculum replicator:

…Day 2

Prepare the **scheme** of the block inoculum replicator including 21 inoculla, the position of 19 test strains and the 2 control strains.

Remember that 20 and 21 positions are the controls.

- Inoculum suspension (10^7 CFU/ml prepared before) in 1.5 mL eppendorf tubes should be ordered in a rack and 500 µl aliquots placed in the corresponding places in the replicator inoculum block.
- Agar plates need to be marked for orientation. ATTENTION VERY IMPORTANT: All the plates must be marked on the same side of the plate to allow to locate strains when reading. Mark the plate and NOT the cover.
- The samples are applied to the agar surface, always orientated in the same way and according to the marking applied, by use of an inocula-replicating device (or standardized loops or pipettes). This protocol is designed to obtain a final concentration of 10^4 CFU/mL.
- A growth control plate (without antimicrobial $MH_{initial}$) is inoculated in the first place and then the plates containing the antimicrobial, starting with the lowest concentration. After all the previous plates are inoculated, a second control plate is inoculated in last place (without antimicrobial MH_{final}) to ensure that there was no contamination or significant antimicrobial carry over during the inoculation.
- A sample of each inoculum is streaked on a suitable agar plate (Blood Agar or Mueller-Hinton agar sterility test) and incubated overnight to detect mixed cultures and to provide freshly isolated colonies in case retesting proves necessary.

The inoculator pins and blocks are disinfected with Virkon for 1 hour (3 litters of water + 2.5 spoons of the powder), then washed thoroughly with distilled water and scrubbed with a swab to guarantee that all disinfectant is removed, otherwise it could affect the growth of bacteria in the following assays. Finally pack and sent to autoclaving after use.

Incubating agar plates:

…Day 2

AEROBIC: The inoculated plates are allowed to stand in a flow chamber until the moisture of inoculum has been absorbed into the agar, but **NO more than 30 minutes**. !!!DO NOT INVERT THE PLATES!!! Incubate at 37º C for 16-24h and then again after 48 hours.

ANAEROBIC ATMOSPHERE: After the inoculum was completely absorbed, incubate the plates in an anaerobic jar (42 plates = 7 litres) with four generators (GENbox anaer – Biomérieux Ref. 36124) and one methylene blue indicator strip (Anaer indicator – Biomériuex Ref. 36118. When incubating in an anaerobic condition is only at 37º C for 20-24h.

Reading and determining agar dilution end points:

...Day 2

After incubation, the plates should be placed on a dark non-reflecting surface to determine end points. The MIC is recorded as the lowest concentration of antimicrobial agent that completely inhibits growth disregarding a single colony or little growth caused by the inoculum.

- If two or more colonies persist after an obvious end point or there is no growth at lower concentrations but it occurs at higher concentrations, culture purity should be checked and the test repeated.
- Register all the results in terms of MIC determined and correspond to tested strains according to the previously prepared scheme for each round of tests and check results of control strains.

Induction assays of *sil* gene cluster

Day 1

1. All induction assays should be performed few days before starting the protocol for the determination of MICs to silver nitrate.
2. Prepare and isolate test cultures and quality control strain cultures (*E. coli* J53 plasmid pMG101) on MHA and check purity before performing the assay. Incubate in the static oven at 37ºC.
3. Prepare BBL™ Mueller-Hinton Broth (BD/reference 212322) for using in all the steps. We will need 10 mL of MHB per test strains plus the controls until the end of the assay.

Day 2

4. Pick up one to two pure colonies of the MHA plates (test isolates + control) and put them into a glass test tube containing 5 mL of MHB. Incubate in a static oven at 37ºC for 14 hours.

Day 3

5. Prepare 10 ml of the stock solution of $AgNO_3$ at 10 mM

$$c = \frac{n}{v} \leftrightarrow 0.010M = \frac{n}{0.010\ L} \leftrightarrow n = 0.0001\ mol$$

$$n = \frac{m}{Mw} \leftrightarrow 0.0001\ mol = \frac{m}{169.87} \leftrightarrow m = 0.016987\ g + 10\ ml\ of\ destilled\ water$$

6. Prepare 10 ml of a solution of $AgNO_3$ at 1 mM in water using the stock at 10 mM

$$Cc\ x\ Vc = Cd\ x\ Cv \leftrightarrow 10\ mM\ x\ Vc = 1\ mM\ x\ 10\ ml$$

$$Vc = 1\ ml\ of\ the\ 10\ mM\ silver\ solution + 9\ ml\ of water$$

7. Pick up the test tubes from the static oven and homogenise the suspension slowly. Take 100 µl from each tube containing the test strains and control, for two new glass test tubes containing:

Tube 1:
100 µl strain +
5 mL MHB

Tube 2 (induction):
100 µl strain +
5 mL MHB +
0.025 mM $AgNO_3$ (use the previously prepared $AgNO_3$ solutions at 1 mM)*

* Prepare 0.025mM $AgNO_3$ using the solution at 1 mM:

$$Cc\ x\ Vc = Cd\ x\ Cv \leftrightarrow 1\ mM\ x\ Vc = 0.025\ mM\ x\ 5\ ml$$

$$Vc = 0.125\ ml\ of\ the\ 1\ mM\ silver\ solution + 4.875\ ml\ of\ MHB$$

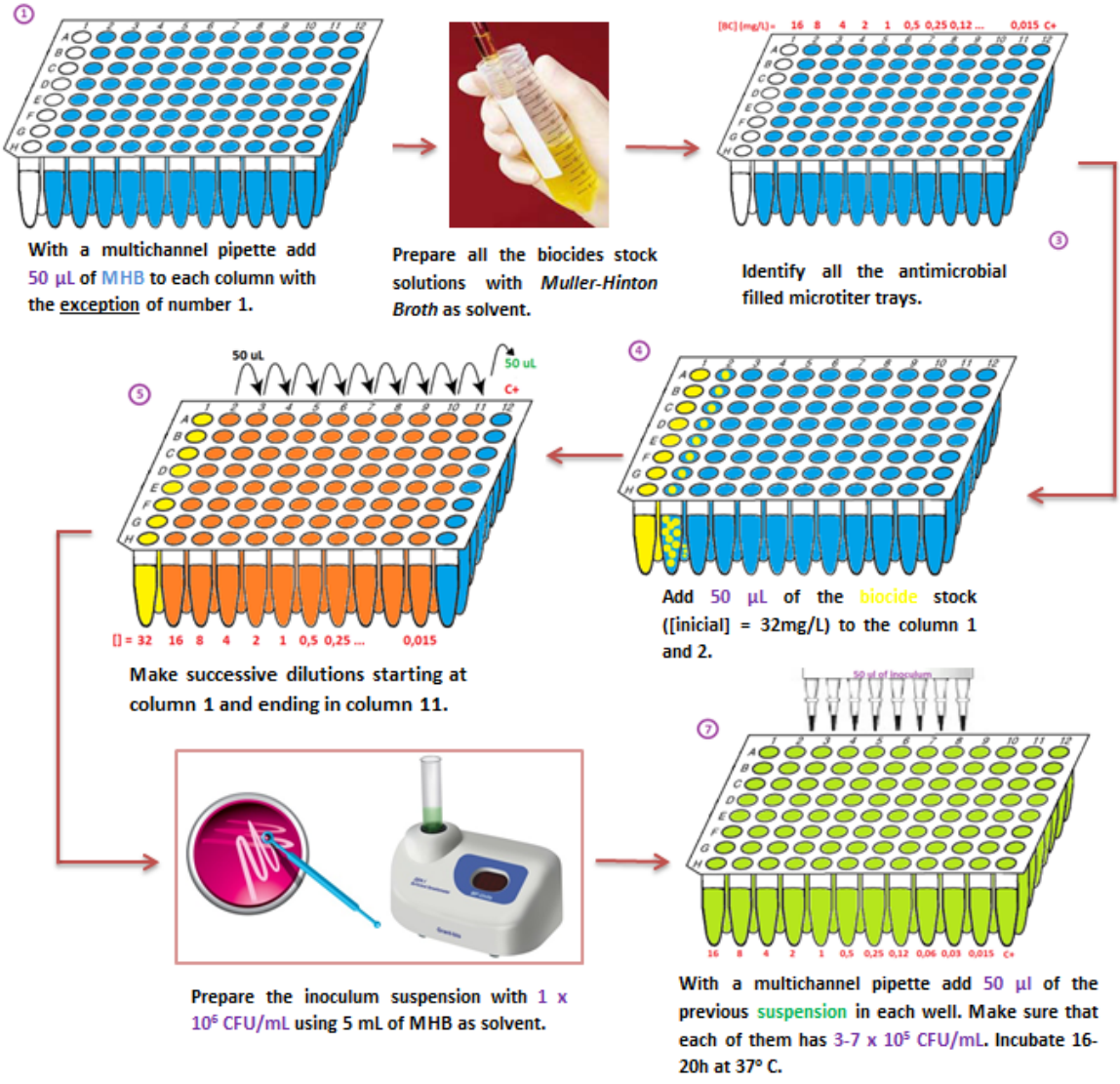
8. Incubate all the previous test tube in a shaking water bath with strong agitation (180 rpm) at 37ºC for 16 hours.

Day 4

9. Pick up the test tube from the shaking water bath and proceed with determination of MICs to $AgNO_3$ following the standard protocol.

Determination of benzalkonium chloride-BZK minimum inhibitory concentrations by the broth microdilution method

The scheme below represents the main steps of the adopted protocol in this work and described in the items below.



Day 1

1. Prepare and isolate test cultures and quality control strain cultures on MHA and check purity before performing the assay.
2. Prepare BBL™ Mueller-Hinton Broth for using in all the steps, taking into account that in each of the 96 wells of the microtiter plate we will have a final volume of 100 µl. Additionally, we will need 20 mL of MHB for the dilution of the stock solution of BZK and 5 mL for each test strain/control.

Day 2

3. Prepare the stock solution of the biocide benzalkonium chloride (powder, B6295-Sigma-Aldrich®) according to the number of isolates that will be tested. In each microtiter plate we can test 6 strains (line A-F) plus the controls (line G-H). In each plate we can use the column 11 and 12 to control the growth of the bacterial suspension and the biocide (without any inoculated strain).

- Concentration range of BZK to use in the assay

Column	1	2	3	4	5	6	7	8	9	10	11	12
Concentration (mg/L)	256	128	64	32	16	8	4	2	1	0.5	C^+	C^-

- Prepare 1 mL of a stock solution of BZK at 100 mg/mL or g/L (Mw = 360 g/mol)

$$M\ (mol/L) = \frac{C\ \left(\frac{g}{L}\right)}{Mw} \leftrightarrow M = \frac{100}{360} \leftrightarrow n = 0.2777777\ mol/L$$

$$c = \frac{n}{v} \leftrightarrow 0.2777777 = \frac{n}{0.001\ L} \leftrightarrow n = 0.0002777\ mol$$

$$n = \frac{m}{Mw} \leftrightarrow 0.0002777\ mol = \frac{m}{360} \leftrightarrow m = 0.099972\ g + 1\ ml\ of\ destilled\ water$$

4. Dilute with MHB the previous stock of BZK at 100 mg/mL in sterile polypropylene tubes to make a final solution at twice the maximum concentration used for the susceptibility testing (e.g. if your maximum concentration of BZK is 256 mg/L you will need to prepare a BZK solution in MHB at 512 mg/L).

- Prepare 20 mL of a MHB solution of BZK at 512 mg/L using the stock at 100 mg/mL

$$Cc\ x\ Vc = Cd\ x\ Cv \leftrightarrow 100\ mg/mL\ x\ Vc = 0.512\ mg/mL\ x\ 20\ ml$$

$$Vc = 0.1024\ ml\ of\ the\ 100\ mg/mL\ BZK\ stock\ solution + 19.8976\ ml\ of\ MHB$$

5. With a multichannel add 50 µl of MHB at each column with the exception of number 1. Then add 50 µl of BZK solution in MHB to columns 1 and 2. With the multichannel make serial dilutions starting at column 2 and ending at the last desired concentration. Discard the last 50 µl of the final concentration.
6. Prepare the inoculum suspension of each strains plus the controls. For the preparation of the inoculum suspension, use 2-3 pure colonies to make a final suspension in sterile saline (NaCl 0,9%) at 0.5 McFarland (1,5 x 10^8 CFU/mL) using a photometric device/nephelometer. Dilute 1:100 the previous suspension to achieve 1 x 10^6 CFU/mL in 5 mL of MHB (e.g. 50 µl of the final suspension in 5 mL of MHB). The last suspension should be distributed (50 µl) in each well of the microtiter plate, optimally within 15 minutes.
7. Incubate the microtiter plate in a static oven at 37°C for 16-20 hours. To maintain the same temperature incubation for all cultures, the trays should not be staked more than 4 highs.

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Publication:	Journal of Antimicrobial Chemotherapy
Publisher:	Oxford University Press
Date:	09/01/2011

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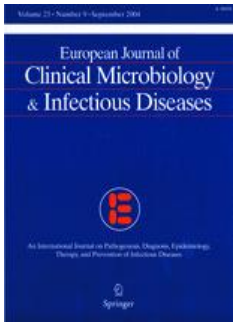

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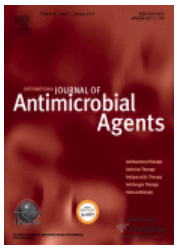

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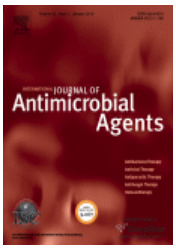

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Publication:	Journal of Antimicrobial Chemotherapy
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